

NITROGEN METABOLISM  
IN PLANTS

*Oxford University Press, Amen House, London E.C.4*

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# NITROGEN METABOLISM IN PLANTS

BY  
H. S. McKEE

CLARENDON PRESS · OXFORD  
1962

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LONDON AND TORRIDGE



TO MY WIFE

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## CHAPTER 1

# THE SOURCES OF NITROGEN FOR PLANTS

### A. General

The atmosphere and the soil are possible sources of nitrogen for plants. The atmosphere has vast reserves of elemental nitrogen, with traces of ammonia and other gaseous nitrogen compounds. Soils contain nitrate, ammonium, and usually organic nitrogen compounds.

It has not always been recognized that nitrogen is essential for plant growth. Van Helmont (1577-1644) published posthumously in 1644 data believed to show that it requires only water. His experiment, carried out at Brussels and famous as an early quantitative study in plant physiology, was described as follows: 'I took an earthen vessel in which I put 200 pounds of soil dried in an oven, then I moistened the soil with rain water and pressed into it a willow shoot weighing 5 pounds. After exactly 5 years there had grown a tree weighing 169

TABLE I (from Woodward, 1899)

<i>Distinction of the soils</i>	<i>The several sorts of water</i>	<i>Weight of the plant when put in</i>	<i>Weight taken out</i>	<i>Weight gained in 56 days</i>	<i>Expense of water</i>	<i>Proportion of the growth of the plant to the expense of water</i>
H	Hyle Park conduit water	127	255	128	14190	1 to 110 $\frac{118}{119}$
I	Hyle Park conduit water	110	249	139	13140	1 to 84 $\frac{141}{140}$
K	Hyle Park conduit water in which dissolved $\frac{1}{4}$ ounces of common garden earth	70	214	108	10731	1 to 63 $\frac{147}{146}$
L	Hyle Park conduit water with the same quantity of garden mould as the former	92	370	284	14950	1 to 52 $\frac{182}{181}$

All weights in grains; 'expense of water' is the amount transpired during the growth of each plant; experiment carried out during summer of 1892.

Somewhat earlier the importance of nitre in plant nutrition was stressed by Glauber (1656) and Mayow (1674). Davy (1836) quoted a statement by Sir Kenelm Digby in 1661 that harley grew very vigorously after being watered with a weak solution of nitre, but dismissed the observation as that of a 'speculative writer'. Glauber found accumulations of nitre in soil impregnated with the excreta of cattle, and concluded that it originated in plants eaten by them. On finding that nitre greatly increased the yield of crops, he proposed it as the 'principle' (chief or sole nutrient) of vegetation. Mayow showed nitre to be present in soils in the spring at the beginning of plant growth, but found none in soils which had supported abundant plant growth. This change he attributed to removal of nitre from the soil by growing plants.

Lemery (1693) attributed to 'a salt resembling saltpetre' the value of manure and other materials used to increase the fertility of soil; he added that such a salt could be extracted from some plants but not from others. Evelyn (1674) stressed the value of saltpetre in the following words: 'I firmly believe that were saltpetre (I mean factitious nitre) to be obtained in plenty, we should have need of but few other composts to meliorate our ground.' Stubbs (1667, 1668), noting that tobacco grown in some parts of Jamaica flashed when smoked, concluded that the ground was full of saltpetre. Sugar cane cultivated in such ground grew bigger and faster than elsewhere, and potatoes (whether *Solanum tuberosum* or *Ipomoea batatas* is not indicated, but the latter seems more likely) matured earlier. Both the sugar cane and the potatoes kept badly and the cane juice did not boil well to sugar. It is interesting that the adverse effects of excessive supplies of nitrate on sugar production were recognized so early; they were confirmed, both with cane and beet, by many later workers, e.g. Barral (1878). The importance of nitre as a plant nutrient was also recognized by Wolff (1723). Stahl (1747) detected nitrate in the green parts of *Fumaria*, *Parietaria*, and *Nicotiana tabacum*.

By 1800 the work of Priestley, Ingenhousz, S  n  bier, and De Saussure established that plants obtained their carbon from atmospheric carbon dioxide. De Saussure (1804) recognized nitrogen as an essential plant constituent, and showed that his experimental plants obtained it from the soil, not from the air. His work marked a great advance in technique, but had little immediate effect on general opinion in agricultural science. Davy (1836) remarked that the nitrogen of plants 'may be suspected to be derived from the atmosphere; but no experiments have been made which prove this; this might easily be instituted upon mushrooms and

substances being liberated by decay of plant material and so passing in a continuous cycle between the plant and its environment. This was valuable exposition of sound though not new ideas; unfortunately Liebig also used his great prestige to support the erroneous theory that atmospheric ammonia was the main source of nitrogen for plants. He postulated a formal analogy between their uptake of carbon and of nitrogen, each being assimilated in gaseous form, carbon as carbon dioxide and nitrogen as ammonia. He held that nitrogen nutrition was identical in all plants, casting quite unjustified doubts on the analytical methods by which Boussingault established the special position of legumes.

Gaseous ammonia at low concentrations is assimilated by nitrogen-deficient plants, their pale yellow-green leaves soon turning dark green (Ville, 1850, 1852; Meyer & Koch, 1873; Schloesing, 1874). Normal air, however, contains insignificant amounts of ammonia (Mulder, 1844; Ville, 1855). Plants derive nitrogen mainly from inorganic compounds in the soil or, by bacterial symbiosis, from the free gas. The need of non-legumes for combined nitrogen in the soil was clearly shown at Rothamsted (Lawes, 1847; Lawes & Gilthert, 1851, 1855), and by Salm-Horstmar (1851) who grew oats in calciaed sand with ammonium nitrate as nitrogen source. He also confirmed the observation (Gris, 1844) that plants require iron for healthy growth, becoming chlorotic in its absence. This demonstration requires good pot-culture technique, the small requirement for iron being easily masked by its absorption from experimental vessels or from salts used to supply other elements.

The assumption that either atmospheric ammonia or organic materials in the soil provided the main source of nitrogen for plants was gradually abandoned during the first half of the nineteenth century. Since that time attention has been focussed on nitrates and ammonium salts as available forms of nitrogen. The absorption of nitrogen is more complicated than that of other essential elements because it is available both as a cation (ammonium) and as an anion (nitrate). The first volume of the *Journal of the Royal Agricultural Society of England* shows the interest of progressive farmers and landowners in artificial nitrogenous fertilizers. Several papers (Barelay, 1840; Daere, 1840; Everitt, 1840; Kimberley, 1840; Zetland, 1840) reported increased yields, usually exceeding in value the cost of the fertilizer and its application, from nitrates in field trials with wheat, oats, turnips, and pastures. 'Gas-water', the washing produced in purifying coal gas, also gave good

Müntz (1889), using soil extracted to remove nitrates and then sterilized, showed that beans (*Vicia*, *Phaseolus*), maize, barley, and hemp (*Cannabis*) assimilated the nitrogen of ammonium salts. No nitrate was found at the end of the experiment in the experimental pots or in controls containing solutions of ammonium salts but no plants. This almost completely excludes the possibility, inherent in earlier work on assimilation of ammonium, that bacteria converted it to nitrate assimilated as fast as it was formed. Good agreement was found between the total nitrogen in mature plants (less the amount in the seeds), and that taken up as ammonia. Treboux (1904) reported similar results with mosses, diatoms, green algae, and *Lemna minor*. Griffiths (1891) and Pitsch (1896) showed that beans absorbed ammonium salts directly in sterile water culture. Mazé (1898a) found ammonium and nitrate equally satisfactory for maize in water culture. Hutchinson & Miller (1909) reviewed much early work on the utilization of ammonium, and demonstrated its direct assimilation in sterile water and sand cultures. Peas grew well with either nitrates or ammonium salts, but wheat did better with nitrates.

More recent work has shown that absorption and assimilation of nitrate and ammonium are sensitive to many environmental factors. Interpretation and comparison of results are thus difficult even in well-controlled experiments. Sterile cultures avoid bacterial activity, but the experimental plants are grown in highly abnormal conditions. In water and sand cultures the volume of nutrient solution is usually small enough for the action of plant roots to change the composition of the medium quite quickly. Concentrations of different ions and their relative abundance at the root surface are thus unstable unless the nutrient solution is replaced continuously or at least changed frequently. Finally, growth of the experimental plants may be limited by some factor other than that under study. In sterile cultures for instance, illumination rather than the nutrients supplied may limit growth. Even in experiments with unicellular algae, where conditions are more readily controlled than for higher plants, effects of pH, illumination, and aeration may obscure comparisons of different sources of nitrogen (Syrett, 1954). As a result of these complicating factors, most conclusions on the availability of different sources of nitrogen, and on their interaction with environmental factors, must be regarded as tentative.

Vauquelin (1809a, b) found much nitrate in leaves of *Nicotiana tabacum* and *Atropa belladonna*, and Braconnot (1827b) in those of sugar-beet. Berthelot (1884) detected it in a wide variety of plants, including a moss (*Hypnum triquetrum*), a horsetail (*Equisetum telmateia*), and a fern (*Pteridium aquilinum*). Molisch (1887) also found nitrate in many species, noting that it was commoner in herbs than in woody plants. The nitrate content of plants varies greatly; very high values are recorded for some species when growing in conditions of ample supply and slow utilization. Boutin (1873, 1874) found up to 22.8 per cent (calculated as potassium nitrate) of the dry weight in *Amarantus atropurpureus*, *A. blitum*, and *A. ruber*. *A. retroflexus* also accumulates nitrate (Woo, 1919); the percentage of total nitrogen occurring as nitrate varies from 1.2 in leaves and 1.8 in seeds to 32.8 in roots, 51.8 in stems, and 56.4 in branches. Berthelot (1884) found the stem to contain most of the nitrate in the plant in *Amarantus*, *Avena sativa*, *Borago officinalis*, and *Triticum sativum* (Table 2). This occurs also in buckwheat (*Fagopyrum esculentum*) and *Bryophyllum calycinum* (Pucher, Wakeman, & Vickery, 1939; Pucher, Leavenworth, Ginter, & Vickery, 1947a, b) and in pineapple (*Ananas comosus*) (Nightingale, 1942a).

TABLE 2

Percentage of total nitrate of plant found in various organs.  
(Calculated from data of Berthelot, 1884.)

Species	Stem	Root	Leaves
<i>Amarantus</i> sp.	79	16	5
<i>Avena sativa</i>	76	22	2
<i>Borago officinalis</i>	76	8	16
<i>Triticum sativum</i>	76	10	14

Nitrate accumulation is reported in sunflower (*Helianthus annuus*) (Nedokuchayer, 1993), celery (*Apium graveolens*) (Platenius, 1931), rye grass (*Lolium perenne*) (Chibnall & Miller, 1931), oats (Sessions & Shive, 1933; Bradley, Eppson, & Beath, 1949; Whitehead, Olson, & Moxon, 1944), wheat (McCalla, 1933), tobacco (Eisenmenger, 1933), and *Salvia reflexa* (Williams & Hines, 1939). Fodder rich in nitrate may poison livestock; the toxic agent is nitrite (Rimington & Quin, 1933; Williams & Hines, 1939) produced by an enzyme of plant origin.



(1953) found them much less favourable for this species than nitrate or some amino-acids.

#### (b) EFFECTS OF pH AND OF NON-NITROGENOUS NUTRIENTS

Many workers found that the pH of the medium affected absorption of both nitrate and ammonium. Plants grown with either nitrate or ammonium change the pH of the medium, solutions with nitrate becoming more alkaline and those with ammonium more acid. The excessive acidity produced by plants supplied with ammonium salts of strong acids was recognized and explained by Rautenberg & Kuhn (1864). A steady pH during the course of an experiment is best obtained by a continuous flow of culture solution, as used by Shive & Stahl (1927) and various later workers (e.g. Street & Roberts, 1952).

The effects of pH on the uptake of nitrate and ammonium have been attributed to changes in the ionic or molecular species present in the medium. This explanation is unlikely to be correct. Nitrate is present as the ion over a wider range of pH than is tolerated by most plant roots. Free nitric acid occurs in significant amounts only at pH levels below 3.0. Ammonium hydroxide molecules, present in neutral and alkaline solutions, have been considered to be the preferentially absorbed form of ammonium. This suggestion, however, fails to explain the high rates of absorption of ammonium observed at pH levels well below neutrality where little ammonium can exist as the hydroxide molecule. Tomato plants, for instance, absorb appreciable amounts of ammonium at pH 4.0 (Clark & Shive, 1934; Arrington & Shive, 1936).

Many workers have concluded that plants use ammonium best at a neutral or alkaline reaction and nitrates in acid media. Results supporting this view are reported for sugar-beet (Prianishnikov, 1929; Dikussar, 1930, 1934), tomato (Tiedjens & Robbins, 1931), and apple trees (Tiedjens & Blake, 1932). Weissman (1951) found that wheat seedlings in the dark gave maximum protein synthesis with equal amounts of nitrogen as nitrate and as ammonium at pH 5.3 and pH 6.3; at pH 4.3 the optimum ratio was one part of nitrogen as ammonium to nine parts as nitrate. Others, however, consider that both nitrate and ammonium can be effectively assimilated over a wide range of pH (Burstöm, 1910; Arnon, Fratzke, & Johnson, 1942; Arnon & Johnson, 1942; Nightingale, 1948). This difference of opinion is due, in part at least, to effects of the total ionic composition of the medium on the assimilation of nitrate and ammonium at different levels of pH.

nutrition are thus variable, and may depend on the species studied.

Among the micronutrient elements whose requirements are affected by the form of nitrogen supplied, molybdenum has been intensively studied; it is associated with enzymatic reduction of nitrate in the mould *Neurospora crassa* and in higher plants (Evans & Nason, 1952, 1953). Tomato and barley (Mulder, 1948), cauliflower (Agarwala, 1952), *Aspergillus niger* (Steinberg, 1937, 1939), and *Anabaena cylindrica* (Wolfe, 1954) all require more molybdenum with nitrate than with ammonium as the source of nitrogen. The importance of manganese in plant nutrition was pointed out earlier (Aso, 1903; Nagaoka, 1904; Loew & Honda, 1904); its association with reduction of nitrate to nitrite and ammonia by plants was stressed by Dony-Hénault (1911, 1912) and by McHargue (1919). A beneficial effect of manganese on nitrate utilization also appears in the results of Plate (1914). Manganese is now known to be essential for assimilation of nitrate in isolated wheat roots (Burström, 1939a, b) and in *Chlorella* (Noack & Pirson, 1939; Alberts-Dietert, 1941). Nitrates accumulate in manganese deficiency in oats (Leeper, 1941; Whitehead & Olson, 1941) and in *Phalaris minor* (Leeper, 1941), suggesting that manganese is required at an early stage in utilization of nitrate. In cauliflower, however, manganese deficiency leads (Hewitt, Jones, & Williams, 1949) to an accumulation of amino-acids, manganese appearing to act at a later stage of the reaction sequence leading from nitrate to protein.

Jones, Shephardson, & Peters (1949) found that manganese prevented an accumulation of nitrite in soybeans grown with nitrate in conditions of inadequate aeration; this recalls the formation of toxic materials from nitrate in pea seedlings grown anaerobically (Godlewski & Polzeniusz, 1901), and suggests an effect of manganese on the reduction of nitrite. The green alga *Ula lactuca* responds to manganese with nitrate but not with ammonium (Kyllin, A., 1943; Kyllin, H., 1943). Manganese stimulates a purified enzyme system from soybean leaves which reduces nitrite to ammonia (Nason, Abraham, & Averbach, 1951). Manganese thus seems essential in the utilization of nitrate; whether it acts at one or more stages remains uncertain. Deficiencies of other elements, e.g. sulphur (Eaton, 1942; Anderson & Spencer, 1950), also lead to an accumulation of nitrate. This probably indicates a general depression of protein synthesis, owing to a deficiency of essential sulphur-containing amino-acids, rather than a direct participation of sulphur or its simple compounds in nitrate reduction.

nitrate is assimilated more readily than ammonium by cotton seedlings grown at low tensions (10 to 15 per cent) of oxygen (Leonard & Pinekard, 1946). In *Bacterium lactis aerogenes* (Lewis & Hinshelwood, 1948) and in excised wheat roots (Nance, 1948, 1950) high concentrations of oxygen interfere with nitrate assimilation; they also inhibit reduction of nitrates by juice from potato tubers (Abelous & Aloy, 1903). The degree of aeration of culture solutions is therefore important in comparing nitrate and ammonium as nitrogen sources.

#### (d) STAGE OF DEVELOPMENT OF THE PLANT

An intense absorption of nitrogen is typical of young plants (Campbell, 1924; Richardson, Trumble, & Shapter, 1932). Prince, Jones, & Shive (1922) showed that seedlings of several species absorbed more ammonium than nitrate from solutions containing both ions. Later in their development this trend was reversed, nitrate being preferentially absorbed. Similar results have been reported by many subsequent workers (e.g. Nafstel, 1931; Sessions & Shive, 1933; Stahl & Shive, 1933*a, b*; Clark & Shive, 1934; Chandler, 1952).

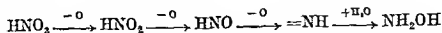
Age effects on the uptake of different forms of inorganic nitrogen have long been studied in rice. Kellner & Sawano (1884) found young rice plants grew better with ammonium than with nitrate, but in later stages of development the position was reversed. This has been confirmed by more recent workers (e.g. Dastur & Malkani, 1933). Many workers have reported better results with ammonium than with nitrate for rice, but in later stages of growth nitrate seems at least equally effective. A similar preference for ammonia in the early stages of growth has been noted for oats (Stahl & Shive, 1933*a, b*) and for maize (Lehmann, 1875). Malavolta (1954), in a brief report, summarized culture studies at pH 6 which showed marked effects of aeration and of molybdenum supply. The best growth was obtained with nitrate plus molybdenum in the absence of aeration. Aeration considerably reduced the rate of growth; its interactions with molybdenum supply and type of nitrogen compound were complex. A metabolic difference was noted between seedlings 4 weeks and 8 weeks old; the former accumulated much ammonia without an increase in amides; the latter had a comparatively high ammonia content but amides were also present. Malavolta (1957) described these results in more detail in a thesis, recording also interesting effects of cyanide on the uptake of nitrate. Addition of potassium cyanide ( $M \times 10^{-4}$ ) to the culture solution inhibited the uptake of nitrate, but not of ammonium or potassium,

require organic sources of nitrogen. A growth response to nitrite remains unexplained; tests with oximino acids did not suggest that it was used by an alternative pathway bypassing ammonia. *Anagallis* embryos used ammonia but not nitrite. Germinating oat embryos use nitrate effectively (Harris, 1956).

#### (e) CARBOHYDRATE STATUS OF THE PLANT

The level of available carbohydrate affects assimilation of inorganic nitrogen. The nitrogen utilized appears mainly as amino-acids, whose carbon chains are derived from photosynthetic products, which also provide energy for nitrate reduction. Ammonia requires no reduction, but unlike nitrate is toxic and must be combined with non-nitrogenous compounds to synthesize useful or at least harmless materials taking part directly in protein synthesis or storing nitrogen for later use. In plants adequately supplied with carbohydrate free ammonia occurs only in traces. The synthesis of amides is considered in detail in Chapter 10; here it may be noted that they are often formed in response to an intake of ammonium. High supplies of ammonium, especially at low light intensities, tend to exhaust carbohydrate reserves. The toxic level of ammonium decreases with light intensity (Mevins & Engel, 1929; Beaumont, Eisenmenger, & Moore, 1933). The main effect of high nitrate supply, apart from nitrate accumulation, is an increased formation of organic acids (Clark, 1936; Wadleigh & Shive, 1939; Vladimirov, 1945; Pucher *et al.*, 1947b). Assimilation of nitrate increases uptake of glucose by *Chlorella pyrenoidosa* in the dark (Thang & Lubochinsky, 1957; Thang, 1959). Some of the extra glucose forms carbon dioxide, but it is mostly used to form the carbon chains of proteins and nucleic acids for which the nitrate supplies nitrogen. No nitrite or ammonia accumulates, and little free amino-acid.

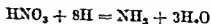
Potato (*Solanum tuberosum*) tubers and eggplant (*S. melongena*) fruits contain a similar enzyme (Abelous & Aloy, 1903; Kastle & Elvove, 1904). Pozzi-Escot (1903) obtained from the stems of burdock (probably *Arctium lappa*) an extract reducing nitrate to nitrite and ammonia. Irving & Hankinson (1908) showed nitrate to be reduced to nitrate in tissues of *Elodea*, *Iris*, *Polamogeton*, *Vallisneria*, *Vicia faba*, and several grasses. Bach (1896) suggested the following stages in reduction of nitrate:



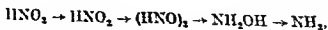
This scheme was based on chemical considerations, without direct evidence for biological occurrence of any stage after the first.

In higher plants nitrate reduction leads in general to assimilation of nitrogen; an exception occurs in cotyledons of *Vigna sesquipedalis*, where nitrate acts as a hydrogen acceptor in anaerobic conditions, though in other parts of the plant only normal assimilation of nitrate is found (Kumada, 1953; Egami, Ohmachi, Iida, & Taniguchi, 1957). Nitrate is an important hydrogen acceptor in many anaerobic bacteria (Quastel, Stephenson, & Whetham, 1925; Stickland, 1931; Woods, 1938; Aubel, 1939; Korsakova, 1941; Lascelles & Still, 1946; Lemoigne, De Somer, & Croson, 1951; McNall & Atkinson, 1956) and for some unicellular green algae (Kessler, 1957a, b). In such cases the nitrogen of nitrate is often not assimilated, being given off as nitrogen, nitrous oxide, nitrite, or ammonia. Several species that reduce nitrate, e.g. *Achromobacterium arcticum* (Rusakova & Butkevich, 1941), *Thiobacillus denitrificans* (Baalsrud & Baalsrud, 1952), *Micrococcus halodenitrificans* (Robinson & Gibbons, 1952), and *M. denitrificans* (Kluyver & Verhoeven, 1954), use it poorly or not at all for synthesis of organic compounds.

Reduction of one molecule of nitrate to ammonia requires eight hydrogen atoms, or eight electrons, according to the equation:



This suggests a four-stage process, as in biological oxido-reductions electrons are usually added or removed in pairs. The most plausible sequence is:



a scheme distinctly resembling that put forward by Bach (1896) on purely chemical grounds. There is now firm evidence that the first and last steps are catalysed by distinct enzymes, whose requirements for

flavin thus precedes molybdenum in the reaction sequence. Nitrate reduction in *Neurospora* requires inorganic phosphate, replaceable by arsenate, selenate, tellurate, or tungstate but not by silicate or adenosine triphosphate (Nicholas & Seawin, 1956; Kinsky & McElroy, 1958). Molybdenum may occur in the enzyme system as phosphomolybdate. A nitrate reductase requiring ferrous iron and ascorbic acid as essential co-factors is reported in tomato roots (Vaidyanathan & Street, 1959).

Several workers (Sato & Niwa, 1952; Baalsrud & Baalsrud, 1954; Kamen & Vernon, 1955; Lenhof, Nicholas, & Kaplan, 1956; Kinsky & McElroy, 1958) associated cytochromes with reduction of nitrate and nitrite. Kinsky & McElroy (1958) found two distinct TPN-cytochrome *c* reductases in *Neurospora*: a constitutive enzyme occurring with any inorganic nitrogen source, and an adaptive enzyme induced by nitrate and involved in its reduction.

### (c) NITRITE REDUCTASE

Yamagata (1940) demonstrated reduction of nitrite by cell-free preparations of *Bacillus pyocyaneus*. Enzymes catalysing its reduction have been isolated from *Neurospora* and soybean leaves (Nason, Abraham, & Averbach, 1954) and from *Azotobacter agile* (Spencer, Takahashi, & Nason, 1957). Like the nitrate reductases they are metalloflavoproteins with FAD as the prosthetic group. The metal involved is uncertain. The first observations suggested manganese, but copper or iron now seems more likely (Nicholas, 1957a; Medina & Nicholas, 1957a). Denitrifying bacteria (see Chapter 5) contain nitrite and nitric oxide reductases; they are flavoprotein enzymes requiring DPNH or TPNH and activated by copper or iron (Najjar & Allen, 1953; Chung & Najjar, 1956a, b).

Silver & McElroy (1954) produced by ultra-violet radiation a *Neurospora* mutant requiring pyridoxine for nitrite reduction. Pyridoxine is a well-known co-enzyme in reactions involving amino-acids, but its precise connexion with nitrite reduction is uncertain. Naphthoquinones related to vitamin K are reported as co-factors of nitrate reductase (Wainwright, 1955; Medina & De Heredia, 1958).

If, as analogy with other biological reductions suggests, nitrite is reduced by a two-electron change, the product must be at the oxidation level of the hypothetical nitroxyl,  $\text{HNO}$ . This has not been isolated; three dimers, hyponitrous acid,  $\text{H}_2\text{N}_2\text{O}_2$ , iminonitric acid,  $\text{HN}=\text{N}(\text{OH})=\text{O}$ , and nitramide,  $\text{NO}_2\text{NH}_2$ , are known, though their chemistry is not as clear as could be wished.

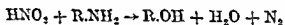
artificial hydrogen carrier (Lascelles & Still, 1946). Anaerobic reduction of nitrate, nitrite, and hydroxylamine occurs in green algae (*Ankistrodesmus braunii*, *Scenedesmus obliquus*) that possess hydrogenase (Kessler, 1957a, b; Damaschke & Lübke, 1958).

## B. The utilization of intermediates in nitrate reduction

### (a) NITRITE

Goppelsroeder (1861) found that sugar beet assimilated nitrite from dilute solutions; higher concentrations damaged the roots. Birner & Lucanus (1866), using oats in water culture, concluded that nitrite nitrogen was not available. Molisch (1887) in careful and detailed studies confirmed that nitrite is used in very low concentrations but at higher concentrations is toxic to roots, and noted its rapid reduction in roots, leafy twigs, and detached leaves of *Primula chinensis*, *Piper macrophyllum*, and *Pelargonium zonale*. Prompt disappearance of  $N^{15}$ -labelled nitrite was observed in wheat leaves (Vanecko & Frear, 1955; Vanecko & Varner, 1955); 82.5 per cent of the nitrite nitrogen absorbed was recovered at the amino level of reduction.

Other workers recorded a loss of nitrogen, probably in gaseous form, from the roots of plants supplied with nitrite (Mazé, 1911b; Mevius & Dikussar, 1930; Mothes, 1938). This loss was attributed to the reaction:

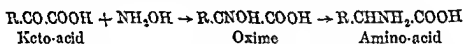


The process would remove toxic nitrite. Pearsall & Billimoria (1937, 1939) recorded large losses of nitrogen from leaves of *Narcissus pseudo-narcissus* floated on sterile nutrient solutions containing nitrate or ammonium. This observation was not confirmed by Mothes (1938), using leaves of *Agapanthus*, *Hippeastrum*, and *Phaseolus multiflorus*, or by Allison & Sterling (1948), who repeated the experiments of Pearsall & Billimoria (1937, 1939) with leaves of *Belemcanda*, *Iris*, and *Narcissus*. Allison, Love, Pinck, & Gaddy (1948) found no loss of nitrogen from *Chlorella* and *Lemna* supplied with nitrogen as ammonia, nitrate, alanine, asparagine, or urea. The reaction of nitrous acid with amino groups to liberate nitrogen requires high acidity and may not be important in physiological conditions. Nonenzymatic reduction of nitrite by ascorbic acid or reduced DPN was studied by Evans & McAuliffe (1956). About 80 per cent of the nitrite nitrogen appeared as nitric oxide; nitrous oxide and free nitrogen were also formed. The reaction was slow at pH 6; its rate rose rapidly with increasing acidity.

Plants vary in sensitivity to nitrite, legumes being more readily

enzymes, e.g. catalase (Keilin & Hartree, 1937) and alcohol dehydrogenase (Kaplan & Ciotti, 1954), containing a free carbonyl group, for which hydroxylamine has a great affinity. Oximes derived from hydroxylamine occur in small amounts in lilac (*Syringa*), *Ampelopsis hederacea*, *Poa pratensis*, *Rumex acetosa*, *Sambucus nigra*, and *Solanum nigrum* (Lemeigne, Monguillon, & Desveaux, 1935, 1937a, b); they are formed also by *Azotobacter* (Virtanen & Järvinen, 1951). Mikhlin (1938) recorded hydroxylamine as a reduction product of nitrite in green plants. The metabolic relations of hydroxylamine are considered in Chapter 3; here it need only be noted that in *Azotobacter* (Virtanen & Järvinen, 1951) and in animal tissues (Yamafuji, Osajima, & Omura, 1960) it appears to arise by both reductive and oxidative processes.

Plants contain several keto-acids, particularly glyoxylic acid, pyruvic acid, oxalacetic acid, and  $\alpha$ -ketoglutaric acid, which could combine with hydroxylamine to form oximes giving amino-acids on reduction:



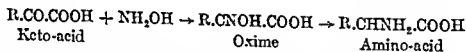
Glyoxylic acid is an early product of photosynthesis; its oxime on reduction would give glycine. Yeast reduces the oxime of pyruvic acid to alanine (Maurer, 1927). The oxime of oxalacetic acid is in some conditions excreted by pea plants (Virtanen & Laine, 1939); it appears also to be an intermediate in the formation of aspartic acid from hydroxylamine and oxalacetic acid by *Clostridium saccharobutylicum* (Cohen & Cohen-Bazire, 1948). Formation of glutamic acid in this way is less likely, as hydroxylamine reacts less readily with  $\alpha$ -ketoglutaric acid than with oxalacetic acid. The yeast *Torulopsis utilis* forms the oxime of  $\alpha$ -ketoglutaric acid when supplied with nitrite, but in smaller amounts than the oximes of glyoxylic, pyruvic, and oxalacetic acids (Virtanen & Saris, 1955). The reduction of oximes requires enzymes different from those reducing hydroxylamine. The oxime of pyruvic acid is not reduced by hydroxylamine reductase; it inhibits reduction of hydroxylamine, apparently forming an unreactive compound with the enzyme (Taniguchi, Mitsui, Nakamura, & Egami, 1955). Kretovich, Bundel, Frasher, & Borovikova (1958), using homogenates of seedling leaves from wheat and pumpkin, found considerable synthesis of serine and glutamic acid from hydroxylamine. Excised tomato roots seemed (Valdyanathan & Street, 1959) to use hydroxylamine; only about a third of that used appeared as ammonia.





enzymes, e.g. catalase (Keilin & Hartree, 1937) and alcohol dehydrogenase (Kaplan & Ciotti, 1954), containing a free carbonyl group, for which hydroxylamine has a great affinity. Oximes derived from hydroxylamine occur in small amounts in lilae (*Syringa*), *Ampelopsis hederacea*, *Poa pratensis*, *Rumex acetosa*, *Sambucus nigra*, and *Solanum nigrum* (Lemoigne, Monguillon, & Desvcaux, 1935, 1937*a, b*); they are formed also by *Azotobacter* (Virtanen & Järvinen, 1951). Mikhlin (1938) recorded hydroxylamine as a reduction product of nitrite in green plants. The metabolic relations of hydroxylamine are considered in Chapter 3; here it need only be noted that in *Azotobacter* (Virtanen & Järvinen, 1951) and in animal tissues (Yamafuji, Osajima, & Omura, 1960) it appears to arise by both reductive and oxidative processes.

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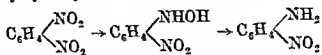


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are metabolized by bacteria, presumably after reduction. Finally, a few nitro compounds occur naturally in micro-organisms and higher plants.

Animal and plant enzymes reduce nitrobenzene to aniline. Gurvich (1941, 1945) found that wheat plants similarly reduced *o*-dinitrobenzene to *o*-nitrophenylhydroxylamine and *o*-nitroaniline:



The reduction, which occurred in the absence of carbon dioxide, was attributed to reducing substances formed by photolysis of water in green tissues. It is not clear why only one of the two nitro groups of nitrobenzene was reduced. Saz & Slie (1954) demonstrated enzymatic reduction of the antibiotic chloramphenicol (chloromycetin, a nitro compound) to an amine by cell-free extracts of *Escherichia coli*. The enzyme was a pyridine nucleotide flavoprotein activated by manganese ions (Saz, Brownell, & Slie, 1956; Saz & Martinez, 1956). Jensen & Gundersen (1955) isolated from soil a form of *Corynebacterium simplex* which broke down nitro compounds, including *p*-nitrophenol, 2,4-dinitrophenol, 4,6-dinitro-*o*-cresol, and picric acid (2,4,6-trinitrophenol). Over half the nitrogen of the dinitro compounds appeared as nitrite. Erikson (1941) isolated from the mud of lakes an actinomycete (*Micromonospora* sp.) that metabolized picric acid and trinitroresorcinol. Species of *Nocardia* use *o*-, *m*-, and *p*-nitrobenzoic acids as sole sources of carbon, nitrogen, and energy (Cartwright & Cain, 1959). The *ortho* and *para* compounds give rise to ammonia, the *meta* compound to nitrite. Little (1957) isolated from pea plants an enzyme system breaking down 2-nitropropane to nitrite and acetone. Rat tissues appear to contain several molybdenum-dependent enzymes, all reducing the nitro group of *p*-nitrobenzenesulphonamide but showing some specificity towards other substrates (Westerfield, Richert, & Higgins, 1957). The metabolic significance of such reductions is not yet clear; the nitro compounds studied may merely be non-specific electron acceptors for flavoprotein enzyme systems.

### C. The effects of light on nitrate assimilation

#### (a) GENERAL

For over a century the possibility of a direct relation between photosynthesis (or some other light-requiring process) and nitrate reduction has engaged the attention of plant physiologists. The effects

of light on the assimilation of nitrate are not yet completely understood, but both green and other organs are known to reduce nitrate. It can be reduced in most plant parts, but the detailed picture varies considerably from one species to another.

Bineau (1856), finding that fresh-water green algae (*Conseria vulgaris*, *Hymenodictyon pentagonale*) took up much more nitrate in the light than in the dark, suggested that photosynthetic processes were involved in the assimilation of nitrate. The moulds *Aspergillus niger*, *Mucor mucedo*, *M. racemosus*, and *Penicillium glaucum* were later shown to use nitrate (Schloesing & Muntz, 1878; Raulin, 1879; Laurent, 1890b). Loew (1890c) argued that protein synthesis in the dark by moulds implied its independence of light in higher plants. He held that in photosynthetic species light affected nitrate reduction indirectly through increased carbohydrate supply and higher respiratory activity. The argument is of dubious value, as nitrate reduction may well follow different courses in mould hyphae and in green leaves. It is also difficult, in a metabolic system involving many interacting pathways, to make any useful distinction between 'direct' and 'indirect' effects.

seems most unlikely to show the distribution of nitrate in living leaves. The distribution recorded for samples analysed about two years after picking is, however, remarkably similar to that found in fresh material by later workers. Schloesing noted that nitrate is neither destroyed nor produced during the processing of tobacco leaf; apparently it is also static, migrating little during drying and fermentation. He analysed separately the midrib and the rest of the leaf (lamina plus lateral veins) for eighteen samples of widely different nitrate content. In each sample the midrib was richer in nitrate than the rest of the leaf. The nitrate content (expressed as per cent nitric acid on the dry weight) ranged from 0.15 to 6.1 in the midribs, and from 0.02 to 1.8 in the rest of the leaf. Nitrate decreased in the midrib with increasing distance from the petiole, and in the lamina with increasing distance from the midrib. Lateral veins had little more nitrate than the lamina.

Schimper (1888) made extensive observations on the distribution of nitrate within the leaf, his results being hidden in a paper whose title mentions only the formation of calcium oxalate. He used a colorimetric method to estimate nitrate in different tissues of the leaf in a wide range of species. The midrib always had more nitrate than the lateral veins, which had in turn more than the mesophyll of the leaf lamina. In nitrate-rich leaves (*Sambucus niger*, *Chenopodium bonus-henricus*, *Hyoscyamus niger*), epidermal cells and leaf hairs had very high nitrate contents. In several species (*Ecballium elaterium*, *Plantago media*, *Taraxacum dens-leonis*) individual tissues within the vascular bundles of the midrib were examined separately, and nitrate was found in the bundle parenchyma rather than in the vascular tissues. Zacharias (1884), however, using microchemical methods, found both nitrate and nitrite in the sieve-tubes of *Cucurbita pepo*.

Schimper (1888) showed that nitrate was consumed in detached leaves of *Sambucus niger*, *Chenopodium bonus-henricus*, *Bryonia dioica*, and *Aesculus hippocastanum*, but not in chlorotic leaves of *Sambucus* or *Aesculus*, nor in non-chlorophyllous parts of variegated leaves of *Alternanthera aurea*, *Fuchsia globosa*, and *Pelargonium zonale*. With green tissues of *Pelargonium zonale* nitrate disappeared in the light but not in the dark. Finally Schimper noted that in several species, including *Acer negundo* and *Taraxacum dens-leonis*, nitrate accumulated much more in shade leaves than in sun leaves. All this evidence is consistent with the view that nitrate coming from the soil is transported into the leaf via the midrib, passes to the chlorophyll-containing cells where it is reduced and its nitrogen used in protein synthesis. Frank (1887a), who

TABLE 3

*Detached leaves of Helianthus; all values are mg protein N/sq m leaf surface. (From Zaleski, 1897.)*

Expt. number	Duration in hours	With nitrate and sugar				With sugar, no nitrate				With nitrate, no sugar			
		Control halves	Expt. halves	Difference	Control halves	Expt. halves	Difference	Control halves	Expt. halves	Control halves	Expt. halves	Difference	Difference
1	0	2621	2853	+232	2014	2010	-4						
2	10	3355	3582	+227	3354	3353	-1						
3	10	2010	2821	+214	2013	2020	+7						
4	18	2140	2640	+194	2451	2457	+6						
9	21							2887	2493			-394	
10	21							2870	2767			-103	
11	21							2823	2578			-245	

placed in the light in nutrient solutions containing nitrate, rapidly synthesized protein, which was deposited in the chloroplasts. Even yellowed nitrogen-deficient leaves formed protein from nitrate if their chloroplasts were not unduly damaged. Stock (1893) recorded similar results for detached leaves of *Achryanthes verschaffeltii* (Amarantaceae).

In *Borago officinalis*, a nitrate-accumulating species, the seed contained 0.3 per cent of nitrate on a dry-weight basis, the young seedling 5 per cent, and the plant a month later 22.6 per cent (Berthelot & André, 1884a). Just before flowering the nitrate content reached 29 per cent; it then fell steeply until in the fruiting stage only 0.3 per cent remained. This suggests that nitrate was used for protein synthesis in the developing seeds; however, it also disappeared in plants prevented from flowering. Molisch (1887) found that detached shoots of *Boehmeria polystachya*, *Goldfussia isophylla*, *Eupatorium adenophorum*, *Hedera helix*, *Selaginella martensii*, and *Tradescantia* sp. retained large amounts of nitrate for several months although many of them put out roots in the culture medium and grew considerably. Nitrate also accumulated in leaves of *Papaver somniferum*, *Rumex sanguineus*, and *Senecio jacobaea* growing in natural conditions (Keegan, 1915, 1916a, b).

Nitrate accumulates in underground storage organs if supplies are high or utilization slow. Its presence in sugar-beet attracted early attention by disturbing the fermentation of beet residues to alcohol (Reiset, 1868; Schloesing, 1868). Barral (1878) found high levels of nitrate (up to 13.9 per cent of the dry weight, calculated as sodium nitrate) in heavily manured sugar-beet, which gave heavy yields of roots containing little sugar. Keegan (1916a) recorded an accumulation of nitrate in winter in the rhizome of the aquatic plant *Menyanthes trifoliata* and in the roots of perennial grasses.

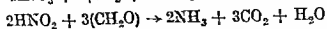
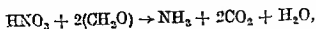
### (c) NITRATE REDUCTION IN ROOTS

Ishizuka (1897) reported that nitrate disappeared during protein synthesis in roots of several species. Many later workers (e.g. Sani, 1929; Burström, 1939b; Nance, 1948) confirmed the disappearance of endogenous nitrate in root homogenates; added nitrate is also consumed. Delwiche (1952), using  $N^{15}$ , showed that nitrate and nitrite are converted to ammonia by cell-free extracts of roots.

In deciduous fruit trees such as apple (Thomas, 1927; Tiedjens, 1934) and peach (Davidson & Shive, 1934; Nightingale, 1935) nitrate occurs mainly in the fine rootlets; it is usually absent from larger roots and from the aerial parts, but reaches the leaves if the soil supply is



expressed formally by the following equations for carbohydrate oxidation coupled with reduction of nitrate and nitrite to ammonia:



In the actual reductions the hydrogen takes part as reduced pyridine nucleotides generated in the respiratory oxidation of carbohydrate.

A close connexion between respiration and nitrate reduction was demonstrated for *Chlorella* by Warburg & Negelein (1920), and has been found also by later workers with unicellular green algae (e.g. Kessler, 1953*a*, *b*). A similar coupling of nitrate reduction to respiration occurs also in higher plants, e.g. barley (Folkes, Willis, & Yemm, 1952) and *Vigna sesquipedalis* (Kumada, 1953; Egami *et al.*, 1957). Reduction and assimilation of nitrite by roots is in some species associated with increased respiration, as in radish (Said & El Shishiny, 1947) and in barley (Yemm & Willis, 1956). In other species, e.g. soybean and wheat (Gilbert & Shive, 1942, 1945; Nance, 1948), oxygen tends to inhibit the reduction of nitrate. The reasons for these differences are not entirely clear. Nitrate reduction in species such as wheat may be coupled to anaerobic fermentative processes, which would also produce the necessary donors of hydrogen. In wheat, reduction of nitrate to nitrite seems to be independent of respiration, but reduction of nitrite to ammonia is coupled to respiration (Nance, 1948). Kessler (1952, 1955) found the reduction of nitrite by *Ankistrodesmus* much more sensitive than that of nitrate to 2,4-dinitrophenol (DNP), which uncouples respiratory phosphorylations from the energy-requiring reactions dependent upon them. This suggests a requirement for energy-rich phosphorylated compounds in the reduction of nitrite.

#### E. General considerations on the reduction of nitrate in relation to other metabolic processes

Nitrate is stable in solution at ordinary temperatures, though subject to photochemical decomposition (Laurent, 1890*a*, *d*; Berthelot, 1898; Thiele, 1907). Its reduction *in vivo* must therefore be coupled to a system providing reducing compounds. The enzymes at present known to participate in the various stages of the reduction of nitrate to ammonia all require reduced pyridine nucleotides, which could arise either in respiration or in photosynthesis. Nothing in this situation implies an obligatory association of any stage in the reduction sequence with photosynthesis. The reductive reactions are in many plant organs

(Walker, 1957; Jolchine, 1959). The phosphoenolpyruvic acid required for this dark assimilation is formed by carboxylation of a photosynthetic product, probably ribulose phosphate. Carbon assimilated in the dark from labelled carbon dioxide appears mainly in malic acid, but also enters several amino-acids, particularly glutamic acid, aspartic acid, alanine,  $\beta$ -alanine, and arginine. Aspartic acid, and hence its decarboxylation product  $\beta$ -alanine, arise by amination of oxalacetic acid. Glutamic acid is formed via the tricarboxylic acid cycle, which is active in these leaves, and leads by decarboxylation to  $\gamma$ -aminobutyric acid. Alanine may arise by amination of pyruvic acid formed by oxidation of malic acid. The presence of labelled arginine suggests an active ornithine cycle.

In the light amino-acids are formed more rapidly than in the dark. The first to appear is alanine, followed by aspartic acid, serine, and glycine. Three of these derive from phosphoglyceric acid, which is directly aminated to alanine and by other reactions yields hydroxypyruvic acid (aminated to serine) and oxalacetic acid (aminated to aspartic acid). Glycine arises from glycolic acid produced in the photosynthetic pentose cycle. Glutamic acid, formed via the tricarboxylic acid cycle, is much more heavily labelled in the light than in the dark. Leucine also appears in much larger amounts in the light. Amino-acids formed in the light but not detected in the dark include methionine, threonine, tyrosine, and valine.

Nitrate increases amino-acid synthesis, as found by Nichiporovich, Andreyeva, Voskresenskaya, Nezgovorova, & Novitzki (1957), and reduces fixation of carbon dioxide. Leaves rich in nitrate fix only 20 to 30 per cent as much carbon dioxide as those with little nitrate. Both in the light and the dark nitrate appears to compete with carbon dioxide for reducing substances.

The literature contains persistent reports of nitrogen fixation by non-nodulated flowering plants. The amounts involved, though often small, would be important in soils low in combined nitrogen. Some such reports lack an adequate experimental basis, but others seem free from obvious errors of technique. Schanderl (1943) claimed appreciable fixation in the absence of root-nodules for many species. Stevenson (1958, 1959) reported small but significant increases of  $N^{15}$  from gaseous nitrogen by shoots (*Coprosma robusta*, Rubiaceae; *Prunus armeniaca*) and roots (*Dactylis glomerata*, *Epilobium erectum*, *Pinus radiata*). Micro-organisms, e.g. bacteria in stipular glands or mycorrhizal fungi, may have performed the actual fixation. Further work in this field is desirable, particularly as pioneer plants often grow vigorously though lacking obvious sources of combined nitrogen.

Symbiotic associations with nitrogen-fixing micro-organisms occur in several unrelated groups of green plants. Nitrogen-fixing blue-green algae form symbioses with fungi (in lichens), liverworts, ferns, cycads, and flowering plants. Associations between flowering plants and micro-organisms which form nitrogen-fixing nodules on their roots occur in many but not all species of the great family Leguminosae, and in a few species of other families scattered apparently at random in the taxonomic system. The families Betulaceae, Casuarinaceae, Coriariaceae, Elaeagnaceae, Myricaceae, and Rhamnaceae contain nodulated species; nodules reported in Zygophyllaceae (Isachenko, 1913; Sabet, 1946; Mostafa & Mahmoud, 1951) and Rubiaceae (Steyaert, 1932) have received comparatively little study. Published statements on root-nodules of Zygophyllaceae are contradictory. Isachenko (1913) found a mycorrhizal fungus with septate hyphae in nodules of *Tribulus terrestris*; he considered that nodulation aided the plant in absorbing water from soils of low moisture content. Sabet (1946) reported nitrogen-fixing bacteria resembling those of the Leguminosae in nodules of *T. alatus* and several other species of Zygophyllaceae. Allen & Allen (1949) found that nodules on the roots of *T. cistoides* contained no endophyte and differed morphologically from those of nitrogen-fixing species.

Some species of Dioscoreaceae (Orr, 1923), Myoporaceae (Stevenson, 1953), Myrsinaceae (Miche, 1911, 1916), Myrtaceae (Stevenson, 1953), and Rubiaceae (Zimmermann, 1902; Boas, 1911; van Faber, 1912, 1914; Rao, 1923; Bremekamp, 1933, 1938) have in their leaves nodules or cavities containing a dense growth of bacteria stated by some authors to fix nitrogen, these species also require further study by modern methods. Bremekamp (1933) listed forty-two bacteriophilous species of

made for combined nitrogen absorbed from rain or from the air. Soils heated to 190°C did not show this increase, suggesting a living agent as responsible for the fixation.

The first organism definitely shown to fix nitrogen was *Clostridium pastorianum*, isolated and described by Winogradsky (1893, 1894, 1902). This anaerobe requires an external supply of carbohydrate. Beijerinck (1901) isolated and described two aerobes, *Azotobacter agilis* and *A. chroococcum*, which he concluded to be nitrogen-fixers because they grew in media to which no combined nitrogen was added. This evidence is inconclusive, as media supposed to be free from nitrogen compounds may contain enough to permit some growth by non-fixing species. However, nitrogen fixation in several species of *Azotobacter* has been amply demonstrated by later workers using more positive methods.

The method of Kjeldahl (1883) was used in most work on nitrogen fixation up to about 1949. The numerous modifications of this method bear witness both to its importance and to difficulties in using it to estimate nitrogen in some biological materials. Various aspects of this method have received systematic study, e.g. by Chihnnall, Rees, & Williams (1943) and by McKenzie & Wallace (1954). The Kjeldahl method gives low values for nitrogen, compared to gasometric methods, with some biological materials and pure organic compounds (Di Frisco, 1929; Lemoigne, Desveaux, & Monguillon, 1934; Anné, 1934; Smyth & Wilson, 1935; Alquier & Sirot, 1937; Wilson, 1939). De Rossi (1935) grew bacterial cultures with organic sources of nitrogen but without access to gaseous nitrogen. The final cultures showed more nitrogen, as estimated by the Kjeldahl method, than the initial media. The absence of gaseous nitrogen excluded bacterial fixation, the apparent increase being attributed to an accumulation of compounds whose nitrogen was fully estimated by the Kjeldahl method.

The amount of nitrogen fixed was often small compared with that originally present in the system studied, so that the results were highly sensitive to sampling errors. The possibility of traces of ammonia or of oxides of nitrogen reaching the culture even in scrubbed air was another source of uncertainty. Some reports of fixation were based only on growth in media stated to be free of combined nitrogen. Very few workers demonstrated a loss of elemental nitrogen from the atmosphere around the culture being tested. Uncertainties regarding chemical methods were often aggravated by doubts about the purity of the cultures studied. It is thus hardly surprising that the more critical students of the subject regarded its literature as infested by unproven

The value of their evidence is enhanced by the fact that they found no fixation by another blue-green alga, *Microcoleus vaginatus* (Oscillatoriaceae). This is probably incapable of fixation, and thus serves as a control for the observations on *Nostoc punctiforme*. The first studies with pure cultures (Pringsheim, 1914a, b) gave negative results but later work (Drewes, 1928; Allison & Morris, 1930; De, 1930; Bortels, 1940; Jensen, 1940; Fogg, 1951; Watanabe, 1951; Williams & Burris, 1952; Kratz & Myers, 1955; Moyse, Coudere, & Garnier, 1957) showed conclusively, in some cases using  $N^{15}$ , that some blue-green algae fix nitrogen vigorously. Nitrogen-fixing species occur in the genera *Anabaena*, *Anabaenopsis*, *Aulosira*, *Calothrix*, *Cylindrospermum*, *Mastigocladus*, *Nostoc*, *Oscillatoria*, and *Tolypothrix* (Fogg & Wolfe, 1954). Some blue-green algae are incapable of fixation. Most species utilize varied nitrogen sources, including ammonia, nitrite, nitrate, amino-acids, and protein. Some use urea (Allen, 1952; Kratz & Myers, 1955). Most species use inorganic sources, but *Synechococcus cedrorum* appears to require organic nitrogen (Allen, 1952). The red-pigmented species *Phormidium persicinum* does not fix nitrogen; it uses nitrate and, somewhat less effectively, ammonia. Organic nitrogen compounds are utilized very selectively. Asparagine is a source of nitrogen, but not aspartic acid, glutamic acid, histidine, or lysine. Organic carbon appears not to be used (Pintner & Provasoli, 1958).

Symbiotic associations are known between blue-green algae and other plants, including the liverworts *Anthoceros* (Leitgeb, 1878), *Blasia* (Waldner, 1879; Molisch, 1925), and *Caricularia* (Molisch, 1925), the floating fern *Azolla* (Strasburger, 1873; Huneke, 1933, Bortels, 1940), several cycads (Reinke, 1879; Schneider, 1894; Life, 1901) and the angiosperm *Gunnera* (Reinke, 1873; Miehle, 1924). Root-nodules of the clover *Trifolium alexandrinum* are reported (Bhaskaran & Venkataraman, 1958) to contain two nitrogen-fixing organisms, a *Rhizobium* and *Nostoc punctiforme*. Several blue-green algae live as endophytes within the large marine green alga *Codium* (Vouk, 1932; Frémy, 1932). Another species occurs regularly in the rhizopod *Paulinella chromatophora* (Lauterborn, 1895; Pascher, 1929). The rhizopod, a unicellular animal, lives autotrophically in association with its algal endophyte.

The algae live in spaces within the host plants, often in their roots. Winter (1935) found that *Nostoc punctiforme* isolated from *Gunnera chilense*, *G. magellanense*, *Cycas circinalis*, *Encephalartos altensteinii* and *E. cycadifolius* fixed nitrogen. Douin (1953) isolated from roots of *Cycas circinalis* and *Stangeria paradoxa* an alga that he considered the

either partner. In most lichens the alga is green, but some contain blue-green algae. Henriksson (1951) reported fixation in culture by a *Nostoc* from the lichen *Collema tenax*. Bond & Scott (1955) demonstrated by the isotopic method that two lichens with blue-green algae fixed nitrogen, in agreement with a conjecture of Ward (1895). Scott (1956) found fixation in *Peltigera praeclata* (containing *Nostoc*) but not in *Cladonia impeza*, which contains a green alga.

Several workers (Sambo, 1923; Henkel & Yuzhakova, 1936; Iskina, 1938) found *Azotobacter* in or upon the thalli of lichens, suggesting its participation in a three-partner symbiosis with their two components. Evidence is, however, lacking that *Azotobacter* is consistently associated with lichens, or transfers nitrogen to them. Krasilnikov (1949) found no *Azotobacter* in many lichens; Scott (1956), using the  $N^{15}$  method, detected no fixation of nitrogen by *Cladonia impeza*, a species stated to contain *Azotobacter*. *Azotobacter* seems, on present evidence, unimportant in the nitrogen economy of lichens.

## B. The biochemistry of biological nitrogen fixation

The biological fixation of nitrogen separates in normal conditions the two strongly united atoms of the nitrogen molecule, a process which industrially requires a large supply of electrical energy. The catalysts acting on the nitrogen molecule in these cells are clearly very efficient; a knowledge of their nature might lead to great improvement of industrial catalysis.

The extensive literature on the biochemistry of nitrogen-fixing organisms is largely irrelevant to fixation *per se*, but contains incidentally much interesting information, including the fact that *Azotobacter* has the highest respiration rate yet recorded (Williams & Wilson, 1954). Nitrogen-fixing organisms may be photosynthetic or saprophytic, aerobic or anaerobic; fixation can thus be superimposed on very different metabolic backgrounds. Detailed knowledge of these backgrounds has great interest and value for comparative biochemistry, but cannot provide much information about the fixation process itself.

The frequent presence of hydrogenase is one of the few uniformities detected beneath the great diversity of metabolic activities among nitrogen-fixers. Hydrogenase catalyzes in either direction the conversion of hydrogen ions to molecular hydrogen. It is widespread among bacteria (Stephenson & Stickland, 1931; Lascelles & Still, 1944, 1946; Phelps & Wilson, 1941). Some micro-organisms in which it occurs do not fix

(Wilson, Burris, & Coffee, 1943) failed, but it was later detected in soybean nodules (Hoch, Little, & Burris, 1957). Rosenhlum & Wilson (1950) reported the rate of anaerobic nitrogen fixation in *Clostridium* to be unaffected by hydrogen, but Hiai, Mori, Hino, & Mori (1957) found competitive inhibition of fixation by hydrogen in *Clostridium*, which contains hydrogenase (Shug, Wilson, Green, & Mabler, 1954). Lee & Wilson (1943) showed hydrogenase formation in *Azotobacter* to be associated with the metabolism of gaseous nitrogen rather than of hydrogen. This finding strongly suggests a connexion between hydrogenase and nitrogen fixation. It was confirmed (Green & Wilson, 1953; Green, Alexander, & Wilson, 1953) by more precise methods than in the original work. Mutant cells incapable of fixation contained little hydrogenase.

It has been suggested that hydrogenase itself or some closely related enzyme catalyses the reduction of nitrogen to ammonia or to some less reduced compound. A preliminary mobilization of hydrogen by hydrogenase, followed by the intervention of another enzyme system to catalyse the interaction of nitrogen and hydrogen, is perhaps more plausible. Hydrogenase, once believed to reduce nitrate, is separable from nitrate reductase in purified systems (Hyndman, Burris, & Wilson, 1953), though the enzymes acting on hydrogen and on nitrate appear to be associated *in vivo*. Similarly, the actions on hydrogen and on nitrogen are probably distinct, though hydrogenase may be associated with some phase of fixation in those nitrogen-fixers that possess it. In some, as noted above, it appears to be absent.

The names 'nitrogenase' and 'azotase' are applied to hypothetical enzymes or enzyme systems catalysing the reduction of molecular nitrogen. They represent little more than the belief, no doubt well-founded, that enzymes take part in fixation. Their study has been greatly hampered by the difficulty of obtaining cell-free extracts or particulate preparations which reliably fix nitrogen. A claim of fixation in cell-free extracts of *Azotobacter* (Bach, Yermoleva, & Stepanian, 1934) aroused much interest, but was not confirmed by later workers (Roberg, 1936; Allison, Hoover, & Minor, 1942; Burris *et al.*, 1943; Imshenetski, Solntseva, Perova, & Kuranova, 1956). Hamilton, Shug, & Wilson (1957) reported changes in the spectra of flavin and cytochrome systems in sonic extracts of *Azotobacter*, *Clostridium*, and soybean nodules after exposure to hydrogen and to nitrogen. These observations strengthen the evidence for a connexion between the metabolism of hydrogen and of nitrogen in these species; they may also represent a

combined nitrogen (Jensen & Betty, 1943). Molybdenum-deficient legumes may be heavily nodulated, but the nodules are inefficient, fixing much less nitrogen per unit weight than those of normal plants (Jensen, 1945; Anderson, 1946; Anderson & Thomas, 1946; Mulder, 1950). Responses to molybdenum by legumes growing in field conditions have been observed by many workers, e.g. Dmitriev, 1939a, b; Anderson, 1946. The seeds of legumes contain relatively large amounts of molybdenum (Bertrand, 1939; Vinogradova, 1943). The molybdenum content of the seed varies considerably in different leguminous species. Seeds of some species of Caesalpiniodeae contain less molybdenum than in other sub-families, but too few species have been examined to indicate whether this is a consistent distinction (Vinogradova, 1953). Molybdenum thus seems to be associated with fixation; its rôle, however, remains unknown and may be indirect. It is essential for flowering plants (Arnon & Stout, 1939; Piper, 1940; Steinberg, 1941) and for fungi (Steinberg, 1936, 1937) which do not fix nitrogen, being involved in the reduction of nitrate to ammonia. The requirement for molybdenum is reduced in plants supplied with ammonium; it may even be eliminated, the element being essential only for plants using nitrate or, if they can do so, molecular nitrogen.

A partial replacement of molybdenum by vanadium is reported for *Azotobacter* (Bortels, 1936; Horner *et al.*, 1942) and for *Clostridium* (Jensen & Spencer, 1947), but vanadium appears ineffective in nodulated legumes (Jensen & Betty, 1943; Anderson & Oertel, 1946; Dmitriev, 1939a, b; Davies & Stockdill, 1956) and in *Anabaena* (Allen, 1956). The vanadium content of whole nodules is comparatively high (Bertrand, 1942). The effectiveness of vanadium in *Azotobacter* was queried by Esposito & Wilson (1956a). Takahashi & Nason (1957) found that tungsten inhibited growth in *Azotobacter* supplied with gaseous nitrogen or with nitrate, the inhibition being reversible by molybdenum. In cultures supplied with ammonium or glutamate the inhibition was much less, and not reversible by molybdenum. Davies & Stockdill (1956) obtained pasture responses suggesting that tungsten replaced molybdenum in symbiotic fixation by legumes. Keeler & Varner (1957) showed that 100 p.p.m. of tungsten in the medium supported growth of *Azotobacter* using nitrate or gaseous nitrogen, though uptake of the radioactive isotope  $\text{Mo}^{99}$  was almost completely inhibited. Keeler & Varner (1958) found no correlation between the uptake and distribution of  $\text{Si}^{31}$  and  $\text{Mo}^{99}$  in *Azotobacter*, which is thus unlikely to metabolize molybdenum as a silicomolybdate complex.



others, require cobalt (Holm-Hansen, Gerloff & Skoog, 1954; Allen, 1956); it does not appear to be associated with nitrogen fixation. The requirement for cobalt is greatly reduced if it is supplied as cobalamin instead of as the cobaltous ion. Levin, Funk, & Tendler (1954) found much more vitamin B<sub>12</sub> in effective nodules of clover, lucerne, and peas than in their roots. Synthesis of the vitamin by rhizobia was demonstrated. Cobalt is reported (Ahmed & Evans, 1959) to stimulate nitrogen fixation in nodulated soybeans, the cobaltous ion being more effective than cobalamin. Reischauer (1960) found cobalt apparently essential for fixation in nodules of lucerne (*Medicago sativa*). Powrie (1960) recorded substantial field responses to cobalt by nodulated subterranean clover.

The effect of combined nitrogen in the medium is complex. Ammonia and substances from which it is readily formed, e.g. urea, inhibit fixation in *Azotobacter vinelandii*, but nitrite and nitrate do so only after a period of adaptation, suggesting that they are first converted to ammonia; aspartic and glutamic acids do not inhibit (Wilson, Hull, & Burris, 1943). Similar results for *A. chroococcum* were reported by Aso, Migita, & Ihda (1939). Both in *Azotobacter* (Newton, Wilson, & Burris, 1953) and *Clostridium* (Zelitch, 1951) comparatively high concentrations of ammonia are needed to inhibit fixation completely. In *Anabaena* the fixation of nitrogen is greatly reduced by ammonium salts or by urea, but is little affected by comparatively large amounts of nitrate (Allen, 1956). Urea and ammonium salts strongly inhibit fixation in *Azotomonas fluorescens*; nitrate, though used by the organism, does not inhibit (Fedorov & Kalininskaya, 1957).

High supplies of combined nitrogen in the soil reduce or even prevent nodulation in legumes, both ammonium compounds and nitrates being effective, as noted by early workers in *Vicia faba* (Rautenberg & Kuhn, 1864; Vines, 1888b), *Trifolium pratense* (De Vries, 1877) and *Pisum* (Laurent, 1901). A 'nitrogen hunger period' occurs when the seedling has used the nitrogen contained in the seed but receives little or no nitrogen from its newly established nodules. Nodulated plants receiving combined nitrogen during this period grow better than those entirely dependent on atmospheric nitrogen. Established soybean plants draw most of their nitrogen from the air even if well supplied with combined forms (Umbreit & Fred, 1936).

Mazé (1898b), pointing out that the free carbohydrate content is low in plants adequately supplied with combined nitrogen but rises in nitrogen deficiency, attributed the better nodulation of deficient plants

root-nodules seem distinct from any of the known animal haemoglobins, but fall within their range of structure. For this reason the name "legbaemoglobin", used for the nodule pigment by Virtanen, Jorma, & Laine (1945) and some other workers, appears unnecessary.

Haemoglobin does not occur in rhizobia growing alone, or in legumes apart from the nodules. This suggests a specific association with fixation, which neither free rhizobia nor non-nodulated legumes can perform. Smith (1949) and Heumann (1952a) reported that in nodules haemoglobin was restricted to large bacteroid-filled cells believed to be the seat of the fixation process, but its rôle in fixation is still obscure. Tove & Wilson (1948) and Virtanen, Jorma, Linkola, & Linnasalmi (1947) were unable to induce fixation in free-living rhizobia by adding nodule haemoglobin. Heumann (1952b), however, stated that rhizobia from pea nodules formed bacteroids and fixed nitrogen in carrot media containing human blood. Confirmation of the latter claim would be of particular interest; several substances produce bacteroids in culture, but fixation in artificial media has not been demonstrated. Haemoglobin may react directly with nitrogen in fixation, but seems more likely to be an oxygen carrier, as in animals. The rhizobia are aerobic and there is evidence (Pietz, 1938; Frazer, 1943) of low oxygen tension in legume nodules.

Combination with haemoglobin may explain inhibition of fixation in legume nodules by low concentrations of carbon monoxide. At higher concentrations it inhibits fixation in *Azotobacter*, *Clostridium*, and *Nostoc*. They have no haemoglobin but contain other haematin compounds with which it may react. Carbon monoxide is an isostere of nitrogen, having almost exactly the same molecular weight, and a similar electronic configuration. It might, therefore, be expected to compete with nitrogen fixation merely by virtue of its physical similarity. Such an inhibition should be competitive, but the inhibition by carbon monoxide in red clover (Lind & Wilson, 1941) and in *Azotobacter* (Ebersole, Guttentag, & Wilson, 1944) appears entirely non-competitive. Animal haemoglobins, though very sensitive to carbon monoxide, are quite unaffected by the high proportion of nitrogen in the atmosphere.

Németh & Matkovic (1957) and Németh (1959) found a yeast (*Saccharomyces* sp.) in nodules of *Lupinus luteus*. It fixed appreciable amounts of nitrogen in culture (2.4 to 5.7 mg N fixed per g glucose consumed, the higher figure being obtained in aerated cultures). Nitrogen was determined by the Kjeldahl method. The fixation required an initial supply of organic nitrogen and was correlated with the

This avoidable confusion is unfortunate, as the problems involved are of considerable intrinsic difficulty.

Most of the known simple molecules containing one or two atoms of nitrogen have been proposed as intermediates in fixation. Formal relations between some of these are shown in Fig. 1. Azim & Roberts (1956a) suggested that fixation is as likely to begin with an oxidation as with a reduction. This view is supported by apparent metabolic similarities between fixation and nitrate assimilation, but there is little direct evidence for it. Labelled nitrous oxide is used by soybean nodules and by *Azotobacter vinelandii*, but only slowly (Mozen & Burris, 1954). Nitrous oxide is a specific competitive inhibitor of fixation in *Azotobacter* (Repaske & Wilson, 1952; Wilson & Roberts, 1954).

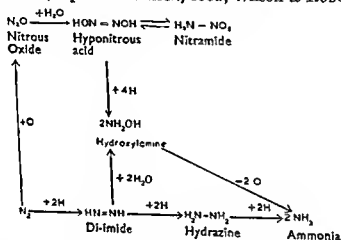


FIG. 1.

It inhibits fixation in *Clostridium* also (Hino, 1955; Lundbom, 1958). At a concentration giving 80 per cent inhibition of fixation it has no effect on uptake of nitrate or ammonium (Mozen, Burris, Lundbom, & Virtanen, 1955). Mozen & Burris (1955) found that *Azotobacter* did not utilize labelled nitramide, which in solution decomposes rapidly to nitrous oxide and water. Evans (1954) detected in rhizobia from soybean, peanut (*Arachis hypogaea*), and two species of *Lespedeza* an enzyme catalysing reduction of nitrate to nitrite by DPNH. Its relation to fixation is obscure, but Cheniac & Evans (1957), using soybean plants inoculated with rhizobia of varying effectiveness, found a positive correlation between nitrate reductase activity and such indices of fixation as haemoglobin in the nodules and total nitrogen in the plants.

Evidence of this type suggests but does not prove participation of oxidized nitrogen compounds in fixation. Oxidized compounds may arise in nitrogen-fixing organisms by minor metabolic pathways rather than

This scheme is chemically plausible. Hydroxylamine forms oximes readily *in vitro* with aldehydes and ketones (Meyer & Janny, 1882). Meyer & Schulze (1884) postulated similar reactions in the plant, suggesting that hydroxylamine could be formed by reduction of nitrate or oxidation of ammonia. They noted the 'aggressive behaviour' of hydroxylamine towards carbonyl compounds, and its 'astonishing facility' in converting them to nitrogenous derivatives. They then supplied hydroxylamine as a source of nitrogen to maize and barley plants, which died in a few days, demonstrating the high toxicity to plant tissues that makes experiments with hydroxylamine difficult. Toxicity of hydroxylamine to plants was confirmed by Loew (1887). Usami (1937) found it toxic in low concentrations to the aquatic moss *Fontinalis antipyretica*. As pointed out by Meyer & Schulze (1884), this toxicity does not rule out hydroxylamine as a possible metabolic intermediate, for *in vitro* it may be utilized without accumulating to toxic levels. Substances with the oxime (CNOH) group occur in the culture medium of *Azotobacter* (Blom, 1931; Endres, 1936). Virtanen & Saris (1955) identified, by reduction to the corresponding amino-acids, the oximes of pyruvic,  $\alpha$ -ketoglutaric, oxalacetic, and glyoxylic acids in the yeast *Torulopsis utilis* after supply of nitrite.

Glutamic acid, originally supposed to be absent from the root excretions of nodulated legumes, was later found in them (Virtanen, Linkola, Hakala, & Rautanen, 1946). This led Virtanen (1947) to suggest that hydroxylamine is reduced mainly to ammonia, which forms glutamic acid with  $\alpha$ -ketoglutaric acid. He thus treated formation of aspartic acid via oxaminosuccinic acid as a minor side reaction, and approached the position of the Wisconsin school that fixed nitrogen entered the dicarboxylic amino-acids and their amides mainly through ammonia.

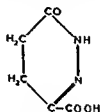
Hydroxylamine reacts more rapidly with oxalacetic acid and pyruvic acid than with  $\alpha$ -ketoglutaric acid (Yamafuji & Akita, 1953). An enzyme reducing oximes to amino compounds occurs in silkworms and other animals (Yamafuji, Kawakami, & Shinohara, 1952; Yamafuji & Omura, 1952) and in the green alga *Scenedesmus* (Yamafuji, Shimamura, & Takahashi, 1955). Yamafuji (1950) reported that silkworms produced oximes from nitrate and ammonium, thus converting inorganic to organic nitrogen. Yamafuji, Osajima, & Omura (1960) and Yamafuji, Osajima, Omura, & Hatano (1960) found in preparations from silkworms and from hen liver, enzyme systems catalysing a series of oxidations and reductions between nitrate and ammonia; they deduced from their data the following metabolic sequences:

The results were broadly similar in all species tested. The highest proportion of  $N^{15}$  always appeared in glutamic acid, usually followed by aspartic acid, alanine, and ammonia (the last including any amide nitrogen present before hydrolysis). Ammonia assimilated by plant cells is largely converted to glutamine and asparagine, which on hydrolysis appear as glutamic and aspartic acids. Aspartic acid also arises from glutamic acid by transamination, or from ammonia by amination of oxalacetic acid. Alanine is formed from glutamic acid by transamination; it also arises from ammonia and pyruvic acid. The distribution of labelled nitrogen supplied as the gas is thus consistent with the ammonia hypothesis, which is further supported by the unchanged distribution in *Azotobacter* supplied with  $N^{15}$ -labelled ammonia (Burris & Wilson, 1946; Burma & Burris, 1957). A culture fixing nitrogen can use ammonia without any lag period; this is consistent with ammonia being formed in fixation. *Clostridium* may excrete into the culture medium up to 50 per cent of the nitrogen fixed, as ammonia, glutamine, and asparagine (Zelitch *et al.*, 1951b). Labelling is very high in excreted ammonia, high in glutamine, and fairly high in asparagine. Nitrogen fixed by cell-free extracts of *Clostridium* appears as ammonia (Carnahan *et al.*, 1969).

In *Alnus glutinosa* (Leaf, Gardner, & Bond, 1958) labelled nitrogen appeared mainly in aspartic acid, glutamic acid, and citrulline, an amino-acid prominent (Miettinen & Virtanen, 1952) in *Alnus*. Citrulline was broken down to ornithine and ammonia, the latter being heavily labelled. These data suggest that in *Alnus* fixed nitrogen passes through ammonia before reaching amino-acids. Citrulline is an important metabolite in the nitrogen-fixing blue-green alga *Nostoc muscorum* (Linko, Holm-Hansen, Bassham, & Calvin, 1957), but has no specific connexion with fixation, being abundant in some non-fixing species. Asparagine was the main amino-acid in root-nodules of *Myrica gale* (Leaf, Gardner, & Bond, 1959); labelled nitrogen appeared mainly in the amide group of glutamine. In both *Myrica* and *Alnus* ammonia was less highly labelled than some other compounds. This led the authors to postulate two metabolic pools of ammonia, only one receiving newly fixed nitrogen directly. They held that their data for non-legumes supported ammonia as the first product of fixation, in agreement with the conclusions of the Wisconsin group for nodulated legumes and non-symbiotic micro-organisms.

One problem thus seems to be settled. Others remain. Little is definitely known, in spite of much speculation, about the steps between

Hydrazine ( $\text{H}_2\text{N}-\text{NH}_2$ ) is another reduced compound postulated as an intermediate without receiving much experimental study, largely because of its toxicity even at low levels (Loew, 1890d). Suzuki & Suzuki (1954) reported oxidation of hydrazine by *Azotobacter* without identifying the reaction products; Riggio-Bevilacqua (1956) made similar observations on pea seedlings. Azim & Roberts (1956b) found hydrazine to inhibit fixation in *Azotobacter* at concentrations above  $2 \times 10^{-5}$  M; at lower concentrations it stimulated fixation, an effect stated not to be due to breakdown to gaseous nitrogen. Bach (1957) supplied hydrazine labelled with  $\text{N}^{15}$  in both nitrogen atoms to *Azotobacter*, and recovered isotopic nitrogen from the cells in three azines,



Dihydropyridazinone-5-carboxylic acid

FIG. 2.

one being probably 3,4-dihydropyridazinone-5-carboxylic acid (Fig. 2). This could arise *in vivo*, as it does (Gabriel, 1909) *in vitro*, by condensation of hydrazine with  $\alpha$ -ketoglutaric acid. The same azines were found in *Azotobacter* grown with gaseous nitrogen and no external supply of hydrazine, and in soybean nodules. They may be directly related to fixation; in *Azotobacter* exposed to labelled gaseous nitrogen they carried more  $\text{N}^{15}$  than either glutamic acid or ammonia; in cells supplied with labelled ammonia they carried comparatively little  $\text{N}^{15}$ . The further metabolism of the azines is not known; on chemical grounds they might yield glutamine or glutamic acid in the cell. Part of their nitrogen could also be released as ammonia.

The intensive use of labelled nitrogen compounds and of chromatographic separation of cell constituents has considerably increased our knowledge of metabolic events related, more or less closely, to fixation. Little, however, is as yet directly known about the process itself. Much of the available evidence suggests a stepwise hydrogenation of nitrogen to di-imide, hydrazine, and ammonia. Di-imide cannot accumulate, being too unstable to have more than a transitory existence, but could be either reduced immediately to hydrazine or combined with water to form hydroxylamine. The position of hydroxyl-

fixation. The mechanisms of fixation may differ in this system and in nitrogen-fixing organisms, but the prominence of molybdenum in both is interesting.

### F. Energy relations of nitrogen fixation

The bond energy of the triple bond between nitrogen atoms is very high. This fact is surprising in two ways. Firstly a high bond energy should, on the generally accepted principles of chemistry, imply great reactivity, yet diatomic nitrogen is notoriously one of the least reactive molecules known. Secondly, the actual bond energy for the  $\text{N}\equiv\text{N}$  bond is much higher than would be expected from the values for the  $\text{N}-\text{N}$  and  $\text{N}=\text{N}$  bonds. This is shown in the table below, values for the corresponding bonds for carbon atoms being given for comparison:

<i>Bond energy (kcal/mole)</i>			
$\text{C}-\text{C}$	82	$\text{N}-\text{N}$	38
$\text{C}=\text{C}$	146	$\text{N}=\text{N}$	98
$\text{C}\equiv\text{C}$	192	$\text{N}\equiv\text{N}$	225

With carbon the increment in bond energy is similar on passing from the single to the double bond, and from the double to the triple bond. With nitrogen the triple bond shows a remarkably large increase in bond energy compared with the double bond. No convincing explanation seems to be available for these anomalies, which suggest that knowledge of the fundamental chemistry of nitrogen is still inadequate. This circumstance must tend to retard progress in understanding the first step in fixation.

It is often stated or tacitly assumed that nitrogen fixation necessarily requires an input of energy from some other process. This view, though firmly entrenched in the literature and repeated by some recent writers, is certainly false. The reduction of nitrogen to ammonia is exothermic (Haber & Van Oordt, 1905) and can therefore proceed without assistance if a suitable mechanism exists. Such a mechanism is clearly found in fixing organisms, which may on balance expend no energy in fixation and are indeed more likely to gain it. The great stability of the  $\text{N}\equiv\text{N}$  bond in the nitrogen molecule is irrelevant to the energy changes occurring once it is broken. Simple thermodynamic arguments, leaving open the nature of the mechanism involved, show the overall process of fixation as yielding rather than consuming free energy if it is essentially a reduction of nitrogen to ammonia.

Christiansen-Weniger (1923) and Burk (1927) concluded that

Bayliss (1956) showed the formation of hydrazine, and still more of hydroxylamine, from gaseous nitrogen to be energetically unfavourable, if coupled to the formation of carbon dioxide from glucose. Such thermodynamic relations do not imply either that a given energetically favourable reaction will occur in any biological system, or that an energetically unfavourable reaction cannot occur. They do, however, show which reactions are feasible without an extra supply of energy, and these may well be regarded as the most likely to occur unless there is evidence to the contrary. Biochemical studies suggest a direct reduction of nitrogen to ammonia in fixation; the thermodynamic data are consistent with this hypothesis which, though not fully proved, is the best available interpretation of the established facts.

### G. Symbiotic nitrogen fixation in legumes

It has long been recognized that leguminous crops enrich the soil when ploughed under as green manures. Descriptions of green manuring, using pulses, clovers, lupins, and lucerne (alfalfa), by the classical writers Varro (first century B.C.) and Columella (first century A.D.) suggest that 2,000 years ago knowledge on the subject reflected long empirical study and observation. Pliny (first century A.D.) stated that 'Everyone agrees that nothing is better for manuring the fields than green lupins ploughed or dug into the ground before the pods are formed', adding that lupins were an excellent substitute for dung and vetches also enriched the soil. Even earlier Theophrastus (370-285 B.C.) wrote that beans seemed to manure the soil and therefore the people of Macedonia and Thessaly turned them into the ground when they were in flower. Early Chinese agricultural writings also mention green manuring with legumes. Virgil in the *Georgics* stressed the benefit of a leguminous crop in a rotation, and recommended sowing wheat after vetches or lupins.

Boussingault (1838a, b, c) found that legumes but not cereals accumulated during their development more nitrogen than was supplied through the roots, indicating its assimilation from the air. His results seem convincing today, but were not so regarded at the time, perhaps because the different behaviour of cereals and legumes remained unexplained. Ville (1855) claimed that both legumes and other plants used gaseous nitrogen. This view was immediately rejected by other workers (Clobez, 1855; Harting, 1855). Boussingault (1855b), finding no significant gain of nitrogen by several legumes and other plants grown in carefully controlled conditions on ignited



essential component of the experimental system, were eliminated by precautions aimed at stray contaminants. The success of these precautions caused the experiment to give an answer which, though correct in the conditions used, was completely false in relation to the question it was planned to study. The whole episode shows that precise and well-controlled experiments which omit an essential factor may mislead while less exact but frequently repeated field observations give a true answer. Schultz-Lipitz (1881), who introduced the terms '*Stickstoffsammler*' (N-accumulator) for legumes and '*Stickstoffresser*' (N-consumer) for cereals, was opposed to the weight of scientific opinion of his day in maintaining that lupins, clover, and peas used a source of nitrogen unavailable to cereals. His conclusion, based on traditional farming practice and on direct observation of enrichment of poor sandy soils by lupins, was nevertheless correct. Reduction of experimental factors to a minimum, a powerful tool in the solution of technical problems, becomes dangerous when some important factor is unwittingly neglected.

## II. Root-nodules of Leguminosae

These nodules attracted the attention of early botanists, being figured without comment for *Vicia faba* by Fuchs in 1542 and described in 1587 by Daléchamps. They are branched structures on the roots of many legumes, including the common cultivated species. Most Leguminosae so far examined possess nodules, but the genera *Adenanthra*, *Bauhinia*, *Cassia*, *Caesalpinia*, *Cercis*, *Ceratonia*, *Gleditschia*, *Gymnocladus*, and *Saraca* contain species in which their absence seems to be normal. The lack of nodules in *Cercis siliquastrum* was noted by Lachmann (1858). Nodules on the stem are rare, but occur in *Aeschynomene indica* (Arora, 1954), which also has numerous root-nodules.

The bacteria forming nodules are not transmitted in the seed, each generation of the host plant being infected from the soil through root-hairs (Ward, 1887) or damaged epidermal cells (Bieberdorf, 1938). Nodules in the aquatic legume *Neptunia oleracea*, which lacks root-hairs, arise by penetration of epidermal cells (Schaefer, 1940). Within the root-hair the bacteria are surrounded (Kny, 1879; Prillieux, 1879) by a thread-like structure passing through the epidermal cells into the cortex of the root, where the bacteria stimulate rapid divisions which form a branched nodule, often large compared with the root bearing it. Development appears to follow release of individual bacteria from the infection thread; it affects both invaded cells and adjacent cells without

phytic species. *Chromobacterium* includes pigmented soil saprophytes. Neither genus is known to include nitrogen-fixtures. Numerous species of *Rhizobium* have been based on specificity towards host plants, but the number of valid species is very doubtful.

The rhizobia are Gram-negative, aerobic rods capable of living saprophytically in the soil, where they usually have a motile flagellated stage. In many leguminous nodules peculiar forms known as bacteroids are prominent, though they are rare in nodules of some species. Brunchorst (1885) coined the term 'bacteroid' for objects which he considered as protein-storing organs of the host cell. They are now recognized as aberrant bacteria, as stated by Frank (1879) and Prillieux (1879). Bacteroids are induced in culture by alkaloids, high acidity, and other special features of the medium. They lack flagellae and are of unusual shapes (X-, Y-, T-, club-, or star-shaped). Many conflicting reports exist on the life-history of rhizobia; more work is needed to clarify the present confused picture. It has been suggested that only bacteroids fix nitrogen, but some effective nodules lack bacteroids, e.g. in *Caragana arborescens* (Allen, Gregory, & Allen, 1955). Bergersen (1955, 1957) considered the morphological features of bacteroids less significant than metabolic changes occurring as they develop from free-living rhizobia. He suggested that bacteria in soybean nodules, though little different in structure from free-living forms, had undergone metabolic changes similar to those postulated for bacteroids.

#### J. Effective and ineffective nodules

Rhizobia isolated from the soil or from nodules vary greatly in ability to induce efficient nodules (Fred, Baldwin, & McCoy, 1932; Virtanen & von Hausen, 1935; Strong, 1937; Purchase & Vincent, 1949; Gregory & Allen, 1953). The effectiveness of a rhizobial strain is unrelated to nodule formation; ineffective strains often induce many small nodules, effective strains forming fewer but larger nodules. Ineffective nodules are small, white, and scattered all over the root system of the host; effective nodules are larger, pink, and mostly on the main roots of the host. Ineffective nodules tend to be round and effective ones elongated by continued growth. Numerous small and necessarily ineffective nodules occur on inoculated legumes grown in atmospheres free from nitrogen (Kossowitsch, 1892; Whiting, 1915).

Variations in both host and rhizobium affect the efficiency of nodules. A rhizobium effective on one legume may be ineffective on another, or

The older work suggests that the nodules fix nitrogen, but this is not yet confirmed by modern methods.

Hooker (1854), in a paper apparently published only as an abstract, described root-nodules in *Podocarpus dacrydioides*, a New Zealand species, and noted their occurrence in *Araucaria*, *Cunninghamia*, *Cupressus*, *Dacrydium*, *Phyllocladus*, *Taxodium*, and *Thuya*. He compared them to the root-nodules of legumes, and suggested that they had some function in the nutrition of the plants that bore them. Van Tieghem (1870) described root-nodules of *Podocarpus neriifolius* as lateral rootlets of arrested growth forming small hemispherical warts arranged in two opposite rows along the roots, and placed so closely as almost to touch one another. Janse (1897) found similar nodules in *P. cupressinus*, but renewed growth of the rootlet formed a series of nodules arranged like a row of beads. The endophyte in these nodules was described as a non-septate filamentous fungus, bearing sporangioles and vesicles, and growing inside the host cells. Nobbe & Hiltner (1899) found a similar fungus in *Podocarpus* nodules, and stated that seedlings without nodules grew very poorly, though nodulated seedlings grew vigorously for five years in a sand free from nitrogen. Shihata (1902) reported that nodules of *Podocarpus chinensis* contained a hyphomycete that assumed an amoeboid form and was finally digested by the host cells. Spratt (1912b) studied nodules from plants of *Podocarpus totara*, *P. elongata*, *P. chilina*, *P. alpina*, *Dacrydium franklini*, *Microcachrys tetragona*, *Phyllocladus trichomanoides*, and *Saxegothaea conspicua* grown at Kew, England. Their nodules were morphologically very similar, and differed from those of other non-legumes (e.g. *Alnus*, *Casuarina*, *Elaeagnus*) in being typically simple structures. Bifurcated nodules were found in *Saxegothaea*, but no species examined bore nodules resembling the much-branched perennial structures of other non-legumes. The podocarp nodules were perennial, a new nodule forming each year inside the old one, in contrast to cycads and non-leguminous angiosperms, where the nodule grows by apical meristems of the branched rootlets.

Spratt (1912b) considered the podocarp nodules to fix nitrogen, and identified the endophyte with the rhizobia of legumes. Fungal hyphae were found rarely and only in the outer parts of nodules. McLuckie (1923a), working in Australia with *Podocarpus spinulosa* and *P. elata*, found the main endophyte to be a bacterium, which he considered similar to but not identical with rhizobias. An intracellular fungus was occasionally present. Yates (1924) studied 20 species of *Podocarpus*

nodules (Spratt, 1912*b*; McLuekie, 1923*a*) also require confirmation.

Little is known of root-nodules in other conifers. Janse (1897) referred briefly to nodules in *Araucaria excelsa*, *Agathis robusta*, *Cupressus fastigiatus*, and *Juniperus chinensis*, all from trees grown in a botanic garden in Java. Yeates (1924) recorded nodules in *Araucaria excelsa* and *Agathis australis*. Both authors considered the endophytes to be filamentous fungi.

#### (b) ANGIOSPERMS

The association of a nitrogen-fixing blue-green alga with several species of *Gunnera* (Halorhagidaceae) has already been mentioned. Root-nodules believed, and in some cases proved, to contain other nitrogen-fixing micro-organisms are known in the dicotyledonous families Betulaceae (*Alnus*), Casuarinaceae (*Casuarina*), Coriariaceae (*Coriaria*), Elaeagnaceae (*Elaeagnus*, *Hippophae*, *Shepherdia*), Myricaceae (*Comptonia*, *Myrica*), and Rhamnaceae (*Ceanothus*, *Discaria*). The total number of species studied is about fifty. Chodat (1904) referred in a very brief report to root-nodules on *Rhamnus* which he apparently considered similar to those of *Alnus* and *Hippophae*. No details were given about the *Rhamnus* nodules nor was it stated on which species of this large and widespread genus they occurred. Root-nodules were recorded in the New Zealand species *Discaria toumatou* by Morrison & Harris (1958), who did not test whether they could fix nitrogen but noted that the family Rhamnaceae, which contains both *Discaria* and the nodulated genus *Ceanothus*, is taxonomically associated with Elaeagnaceae, all three genera of which are known to include species with nitrogen-fixing root-nodules. *Dryas drummondii* (Rosaceae) growing in Alaska has root-nodules considered from field observations to be capable of nitrogen fixation (Lawrence, 1953; Crocker & Major, 1955; Cooke & Lawrence, 1959). Montemartini (1906) reported root-nodules in *Daliscia cannabina*, a member of a small family (Datisceae) of doubtful systematic position but not closely related to any family containing known nitrogen-fixing species. The nodules were stated to contain bacteria resembling those of leguminous root-nodules; their ability to fix nitrogen seems not to have been tested. MacDougal (1894) stated that *Isopyrum bilernatum* (Ranunculaceae) had nitrogen-fixing root nodules, but in a later paper (MacDougal, 1896) he appeared to abandon this claim.

Records of root-nodules among monocotyledons are few and inconclusive. A report (Nogter, 1939) of fixation in root-nodules of the

nodules or nodulated plants of *Alnus*, *Myrica*, and *Hippophae* (Bond, 1955), *Cosuorino*, *Ceanothus*, and *Shepherdia* (Bond, 1957b), and *Coriaria* (Stevenson, 1958; Harris & Morrison, 1958). Bond (1956a) used  $N^{15}$  to demonstrate fixation by nodules still attached to roots of *Alnus glutinosa* growing in natural habitats.

These results confirm that root-nodules in these genera are similar in function to those of the Leguminosae. The nature of their endophytes remains obscure. Varied views have been held on this subject; some workers have successively supported several theories strikingly at variance with one another. There is no reason to suppose that all non-legume nodules contain similar endophytes. Cross-inoculation occurs between the three genera of Elaeagnaceae, though some possible combinations seem not to have been tested (Roberg, 1934; Gardner & Bond, 1957), but not between *Alnus* and *Elaeagnus*, *Hippophae*, or *Myrica* (Roberg, 1934; Bond, Fletcher, & Ferguson, 1954).

Woronin (1866, 1867) described the *Alnus* endophyte as a non-septate filamentous hyphomycete with terminal vesicles, and named it *Schinzia alni*. Warming (1876) recorded root-nodules in *Elaeagnus*, *Hippophae*, and *Shepherdia*. He noted the resemblance of the *Hippophae* nodules to those of *Alnus* but held the endophyte to be a myxomycete similar to *Plasmodiophora brassicae*, described by Woronin (1875) as causing club-root in cabbage and other crucifers. This view, supported by Gravis (1879) and Schroeter (1889), was accepted by Woronin (1885); the names *Plasmodiophora alni* (Moeller, 1885) and *P. elaeagni* (Schroeter, 1897) were proposed for the endophytes. Bruncborst (1886), however, maintained that the *Alnus* organism was a filamentous fungus (*Frankia subtilis*) related to the Mucorales. Moeller (1890) switched his preference to *Frankia subtilis*, but Frank (1887b), renouncing the honour of having this lowly but controversial object named after him, declared that it was not an organism at all but a protein-storing organ of the host cell. He proposed to delete *Schinzia alni*, *Plasmodiophora alni*, and *Frankia subtilis* from mycology ('aus der Mykologie zu streichen') and added for good measure *Schinzia leguminosarum*, a name then used for the rhizobia of legumes. Later (Frank, 1891) he suggested that the organism (as he again regarded it) was related to the filamentous bacterium *Leptothrix*. Further names were proposed in bacterial genera: *Streptothrix* (Hiltner, 1898); *Mycobacterium* (Shibata, 1902; Peklo, 1909); *Frankiella* (Maire & Tison, 1909); *Rhizobacterium* (Dangeard & Leclitova-Trnka, 1909); *Rhizolobium* (Panosyan, 1943). Other workers (Roberg, 1934, 1938; Schlaede, 1933, 1939; Fletcher, 1955) also regarded

Plasmodiophorales and probably to the genus *Plasmodiophora*. They doubted if the true nodule organisms had ever been cultivated on artificial media. Quispel (1954a, b) also held the endophyte of *Alnus* to be incapable of cultivation on any media hitherto tried, and agreed with Krebber (1932) and Bouwens (1943) that its nature remained unknown.

Pommer (1956) found that nodule formation in *Alnus glutinosa* was initiated by an actinomycete that entered root-hairs and stimulated the root tissues to active cell division. Two mould fungi (*Cylindrocarpum radicola* and *Penicillium albidum*) induced nodules indistinguishable in their early stages from those produced by the actinomycete. These fungal nodules were short-lived, in contrast to the perennial nodules formed in natural conditions; none survived more than twelve weeks, the host plant promptly cutting off the infected tissue by a periderm. The similarity between early stages of the nodules induced by moulds and by actinomycetes may thus be only superficial, the final reaction of the host being different. It is nevertheless of great interest that nodule formation can be initiated by pathogenic fungi. Pommer (1956) grew in artificial media an actinomycete isolated from *Alnus* nodules, but in repeated trials was unable to induce nodules in plants inoculated with it.

Pommer (1959) reported isolating from root-nodules of *Alnus glutinosa* a fungus inducing typical nodules when inoculated into seedlings of the same species grown in sterile culture on silica gel. The organism was very different from *Actinomyces alni* Peklo. When cultivated on glucosc-asparagine agar it produced a narrow non-septate mycelium with short branches bearing terminal vesicles. This part of the description strongly recalls that of Woronin (1866); Zach (1908) also reported a filamentous fungus in *Alnus* nodules. Some hyphae became septate and formed bodies named 'bacteroids', though their relation to the objects so named in leguminous root-nodules is quite obscure. The septate hyphae then developed numerous swellings, often on short lateral branches, which grew into large vesicles packed with 'bacteroids'. These bodies, figured also by Schaede (1933), were regarded as spores, but germination was not established. Septate hyphae and vesicles with 'bacteroids' were found in root-nodules of *Alnus glutinosa* as well as in culture. The endophytic fungus was not named nor was a systematic position assigned to it. Cultivation of similar endophytes from root-nodules of *Elaeagnus umbellata*, *Hippophae rhamnoides*, and *Shepherdia argentea* was also reported, but no details

nodulated plants and excised nodules of *Casuarina cunninghamiana* by the isotopic method (Bond, 1957b).

The nodules of *Ceanothus* have not been much studied though they have been recorded in *C. americanus*, *C. azureus*, *C. delilianus*, *C. fendleri*, *C. microphyllus*, and *C. ovatus* (Arzberger, 1910) and in *C. cordulatus*, *C. diversifolius*, *C. fresnensis*, *C. impressus*, *C. integerrimus*, *C. parrifolius*, and *C. prostratus* (Quick, 1944). Atkinson (1891, 1892) referred the causal organism to the Plasmodiophorales, pointing out its similarity to the endophyte of *Alnus* and also to *Plasmodiophora brassicae*. Bottomley (1915) found, as usual, bacteria like legume rhizobia but fixing nitrogen vigorously in culture. Nodules are absent on *Ceanothus* plants cultivated as ornamentals in Britain (Bottomley, 1915; Hawker & Fraymouth, 1951), even in species that are nodulated in North America, the home of the genus. The absence in British soils of the nodulating organism for *Ceanothus* suggests that it is distinct from those of *Alnus*, *Hippophae*, and *Myrica*, and *a fortiori* from the rhizobia of the Leguminosae. American species of *Myrica* cultivated in France form nodules (Chevalier, 1902); they may thus share the endophyte of the European species, *M. gale*, which occurs also in North America. Some other species produce nodules when planted outside the natural area of the genus to which they belong. Several species of *Casuarina*, a genus not occurring naturally in America, are consistently nodulated in Florida (Mowry, 1933), and probably also in Central America and the West Indies. *Casuarina* appears to lack nodules in European botanic gardens and in Egypt (Miehe, 1918; Bond, 1957a). Sydow (1924) recorded *Plasmodiophora elaeagni* from roots (presumably root-nodules, but this is not stated) of *Elaeagnus japonica* cultivated in New Zealand, where the genus is not native.

#### L. Fixation in detached root-nodules

Most early attempts to demonstrate fixation in detached nodules had dubious or frankly negative results. Krashenninnikov (1916), in a paper not widely available but summarized by Wilson (1940), recorded changes in the nitrogen content of atmospheres around detached nodules, and reported fixation in sixteen out of twenty-one experiments at high oxygen tensions. Many subsequent workers obtained no fixation by detached nodules, e.g. Beijerinck (1918), who used samples of up to 1 kg of lucerne nodules, Galestin (1933) and Hurwitz & Wilson (1940), using a sensitive gasometric method.

The first attempts to demonstrate uptake of  $N^{15}$  by detached nodules

continuously transferred nitrogenous compounds to the host throughout their life. Cytological observations showed that the bacteria disintegrated about the same time as the general cellular collapse in the senescent nodule (Dangeard, 1926; Milovidov, 1928; Hocquette, 1930). This disintegration, which may indicate digestion of bacteria by host cells, does not always occur. Thornton (1930, 1936) found rhizobia invading the intercellular spaces and the middle lamellae of the cell walls, and suggested that they became parasitic in senescent nodules. Even if digestion does occur it is relevant only to the final evacuation of nitrogen from senescent nodules. Transfer of nitrogen clearly begins much earlier, as benefits from nodulation appear in young seedlings before any nodules are senescent. Here transfer must occur in some other way. Presumably the bacteria excrete nitrogenous compounds, which are then absorbed by the host cells of the nodules and transferred to other parts of the plant.

Bond (1936) showed that in soybean a very high proportion of the nitrogen fixed, probably 80 to 90 per cent, is regularly exported from the nodules to other parts of the host plant. Similar results are recorded for other legumes (Jensen, 1948; Virtanen, 1952), and for *Alnus glutinosa* (Bond, 1936b), in which fixed nitrogen moves to the shoot in the xylem. Wilson & Umbreit (1937a) distinguished three phases in relation to the transfer of nitrogen from nodule to host plant in soybean. Young and actively growing nodules retain a comparatively high proportion (up to 50 per cent) of the nitrogen fixed. This phase does not last long, and during the main growth period of the plant transfer accounts, as found by Bond (1936), for 80 to 90 per cent of the nitrogen fixed. In the final stage, when the host plant is flowering and fruiting, transfer equals or exceeds fixation, the comparatively small amount stored in the nodules being evacuated. This phase is well seen in the data of Bond (1936).

Flowering and fruiting of the host are often associated with degeneration and shedding of nodules (Tschirch, 1887; Wilson, 1931). There is some evidence that this is a hormonal effect. The shedding of nodules in *Vicia sativa* can be delayed by removing flower buds from the host plant (Pate, 1958b). Ali-Zade (1941) recorded data suggesting that the host plant controls protein metabolism in the nodules through a hormonal mechanism. He found with *Lupinus luteus* that synthesis predominated in preparations from nodules of plants at the early flower-bud stage, and hydrolysis when the plants were flowering. The predominance of hydrolysis was still more marked in nodules from



with the amounts of nitrogen removed by cropping have rarely been recorded. Jensen (1940), in an extensive and careful study of the nitrogen economy in soils of the New South Wales wheat belt, found *Azotobacter* in 50 per cent of the soils of pH 6.0 or above. Most soils had very few *Azotobacter*; only 5 per cent gave counts above 600 per g. Swaby (1939) recorded similar results for the wheat belt of Victoria. McKnight (1950) found black earth soils in Queensland to be rich in *Azotobacter*, but it was usually absent in poor soils derived from granite or coastal sands. Nitrogen-fixing species of *Clostridium* were present in 140 out of 143 soils tested. Tehan & Beadle (1955) estimated the maximum possible contribution by *Azotobacter* to the nitrogen capital of arid soils in Western New South Wales at 0.1 lb/acre/year (0.1 kg/ha/year), compared with 3 lb/acre/year (3.4 kg/ha/year) by blue-green alga. These amounts are very low but may be significant in areas where the annual loss of nitrogen is also low.

Swaby (1939) and Jensen (1940) found little *Azotobacter* in soils of pH below 6.0. Later workers have, however, found *Azotobacter* species flourishing at pH values between 4 and 5 in Australia (Tehan, 1953a: *Azotobacter beijerinckii* var. *acidotolerans*), Denmark (Jensen, 1955: *A. macrocytogenes*) and England (Metcalf *et al.*, 1954: *Azotobacter* spp.) Bacteria fixing nitrogen are widespread in acid tropical soils (Altson, 1936; Starkey & De, 1939; Kaufmann & Toussaint, 1951) and may be important in their nitrogen economy. These species are now referred (Derx, 1956; Tehan, 1953b, c, 1957) to the genus *Beijerinckia*. Ruinen (1956) found it to abound on the leaves of forest trees and epiphytes in Java. Roy & Mukherjee (1957) described another tropical acid-tolerant nitrogen-fixing bacterium, whose growth was inhibited by both nitrate and ammonium; they did not name the organism but considered it distinct from *Azotobacter*. Extra-tropical occurrences of *Beijerinckia* are reported in Japan by Suto (1957) and in South Africa by Becking (1959), who suggested that it is associated with lateritic soils rather than with tropical climates.

Some authors (e.g. Demidenko & Timofeyeva, 1937b) claimed that in the rhizosphere (the soil close to plant roots) *Azotobacter* is much more abundant than in the general soil mass, but Jensen (1940) found no evidence of this with wheat plants in Australia. In a series of 264 agricultural soils in Denmark (Jensen, 1950b) 73 per cent had less than 100 *Azotobacter* per g, 93 per cent less than 1,000 per g, and 99 per cent less than 10,000 per g. The numbers of *Azotobacter* in most soils thus seem inadequate for significant fixation. Other factors also limit its activity.

basically is agar but a cell-wall material from algae?' Pshenin (1959) found *Azotobacter* always present, though in variable numbers, in sediments on the bottom of the Black Sea at depths from 10 to 2,200 m. The main species was *A. chroococcum*; *A. agile*, *A. insigne*, *A. nigricans* and *A. vinelandii* were also recorded. Nitrogen fixation by *Azotobacter* in fresh and marine waters may well be significant, but the available data hardly permit an estimate of its importance in the nitrogen cycle as a whole.

(ii) *Blue-green algae (Cyanophyceae)*. Blue-green algae have an almost ubiquitous distribution and could be important in the economy of nitrogen if many species prove capable of fixation. As photosynthetic organisms they seem likely to flourish in soil only at or near the surface. Some species, however, use organic compounds and may live saprophytically at greater depths. Blue-green algae are found in waters of all temperatures from hot springs, the habitat of the nitrogen-fixing species *Mastigocladus laminosus* and *Oscillatoria subbrevis*, to the cold lakes of the Arctic and Antarctic. They are prominent in fresh water, salt marshes and the intertidal zone. Some marine Cyanophyceae are red or purple in colour, occurring at depths of 30 m or more and flourishing at low light intensities. A red-pigmented Cyanophyceae, *Trichodesmium erythraeum*, appears periodically in vast numbers at the surface of the sea, producing a discoloration stated to have inspired the names of the Red Sea, and the Vermilion Sea (Mexico). *T. erythraeum* probably normally grows at a considerable depth, becoming detached at times and floating to the surface (Feldman, 1932; Pintner & Provasoli, 1958).

Like other algae, the Cyanophyceae occur typically in moist habitats; they are abundant in soils of pasture and cultivated land in Scotland (Fenton, 1943). Some species resist desiccation and are found in arid soils, where with other algae and lichens they form a surface crust in which nitrogen accumulates (Shields, Mitchell, & Drouet, 1957). Algae growing in dry situations may be metabolically active only for short periods after rain. Cyanophyceae are pioneer occupants of newly exposed surfaces; they occupy many specialized ecological niches, boring into shells and limestone rocks, or growing under quartz pebbles in arid country. Treub (1888) visited the island of Krakatau, off Java, three years after the volcanic eruption that destroyed its vegetation and buried the former surface beneath a layer of ash and pumice one to many metres thick. The most conspicuous colonists of the newly formed surface were ferns, but Treub concluded that their spores were able to germinate only because the ash and pumice were covered

of the first seasons, but in the pots with darkened soil the yield fell. Over the five years there was a marked increase in the nitrogen content of the soils with abundant algae, and a decrease in the soils where they were absent. The luxuriant growth of blue-green algae in this experiment, and in rice fields, is attributed by the authors to the high carbon dioxide supply from the respiring rice roots, and in the later years also from decomposition of root residues in the soil. Even in these favourable conditions the algae did not benefit the rice during the first three years. This suggests a transfer of nitrogen to the rice after decomposition of the algae rather than by excretion. More rapid increases in growth and yield of rice grown with *Tolypothrix tenuis* were reported by Watanabe, Nishigaki, & Konishi (1951).

De & Mandal (1956) used a gasometric method to test fixation in six rice soils in pots under water-logged conditions. They estimated the gain in nitrogen from fixation by blue-green algae over six weeks at 14 to 44 lb N/acre (16 to 49 kg N/ha); with added phosphate and molybdenum the best soil gained 70 lb N/acre (78 kg N/ha). Venkataraman, Dutta, & Natarajan (1959) showed *Cylindrospermum sphaerica*, common in cultivated soils near Delhi, to be an effective nitrogen fixer. Nitrogen fixation by blue-green algae may be appreciable in fresh-water lakes; high rates of fixation probably occur for short periods only (Aleyev & Mudretsova, 1937; Hutchinson, 1941; Dugdale, Dugdale, Neess, & Goring, 1959).

The nitrogen-fixing species *Anabaena cylindrica* excretes large amounts of polypeptides in culture. These rather complex compounds may not be directly available to higher plants; they are largely unavailable to the green alga *Chlorella* and to *Anabaena* itself (Fogg, 1952). *Azotobacter agile* grown with fumarate excreted over 50 per cent of the nitrogen fixed, mostly in organic compounds (Fedorov, 1952). The mould *Scopulariopsis brevicaulis* excreted 50 per cent of the nitrogen taken up as nitrate or ammonium in peptides that it could not re-utilize, though it used the constituent amino-acids (Morton & Broadbent, 1955). Similar results are reported for *Aspergillus niger* (Ivanov & Osnitskaya, 1934) and for yeasts (Ivanov & Krupkina, 1929; Reindel & Hoppe, 1952). Both fixing and non-fixing micro-organisms may thus excrete appreciable amounts of nitrogen, a physiological resemblance to animals rather than to higher plants.

(iii) *Other photosynthetic micro-organisms.* Several photosynthetic bacteria fix nitrogen (Lindstrom, Burris, & Wilson, 1949; Lindstrom, Tore, & Wilson, 1950; Lindstrom, Lewis, & Pinsky, 1951). Little is

(2) *Rhizobia symbiotic with legumes*

The Leguminosae are one of the most numerous plant families, with about 12,000 species, including herbs, shrubs, climbers, and large forest trees. The family, though almost cosmopolitan, is represented in temperate regions mainly by herbs, woody legumes being typical of warm climates. Legumes, though often prominent in natural vegetation, are inconspicuous in some areas. In New Zealand, for instance, they form only a minor part of the native vegetation; the highly productive pastures of that country are, however, based on introduced clovers.

De Candolle (1855) and Andrews (1914) considered the Leguminosae as basically a family of trees and woody climbers, which arose in the tropics and spread later into temperate and even cold regions. The Leguminosae were already present in the Cretaceous, when climatic conditions resembling those now found in the wet tropics covered a large part of the earth. Subsequent climatic changes restricted such conditions to the comparatively small area enjoying them today. Two sub-families of the Leguminosae, Mimoseae and Caesalpinioideae, are largely tropical, with some extensions to warm temperate regions. Many genera and very numerous species of the third sub-family (Papilionatae) are shrubs, slender climbers, and herbs adapted to temperate and cool conditions. Most of the legumes familiar as temperate crops and pasture plants belong to the wholly temperate tribes Trifolieae and Vicieae of Papilionatae; a few belong to Phaseoleae, a mainly tropical tribe of the same sub-family.

About 90 per cent of the legumes examined possess nodules, but information is available only for a small minority of the species. Fixation is definitely known in still fewer species, but may reasonably be assumed to occur in any nodulated legume. Caesalpinioideae seem on the scanty evidence now available to have relatively more non-nodulated species than the other sub-families. Some genera, e.g. *Cassia* (Leonard, 1925), contain both nodulated and non-nodulated species. Rather few tropical legumes have been examined for nodules, especially when growing in natural conditions. There are obvious reasons for this unfortunate position. The species involved are very numerous, and many grow in areas difficult of access. Adequate study of the roots of a tree or large climber is slow and laborious. Species growing in a seasonal climate may be nodulated at one time of year (probably the wet season, which hampers investigation) and not at others. Such

Australian legumes in 48 genera, the total number of known Australian species being about 1,100 in 101 genera.

Norris (1956) pointed out that ideas on the mineral requirements of leguminous crops are based on the study of comparatively few temperate species, all belonging to the tribes Trifolieae and Viciaeae. Most leguminous temperate crops demand fertile soils. They require large supplies of calcium and phosphorus, and show little tolerance for acid soils. It cannot be expected that these requirements will be shared by tropical species, as tropical soils are in general acid, highly leached, and deficient in calcium and phosphorus. Most tropical legumes which have been examined are nodulated by rhizobia of the 'cowpea type'; they do not show the high host-rhizobium specificity found in temperate legumes. Norris (1956) associated this specificity, together with high mineral requirements and intolerance of acid soils, with specialized and recently evolved temperate species. In the Leguminosae as a whole low mineral requirements and a low degree of rhizobial specificity, as found in the tropical species, are much more usual.

In sub-tropical Queensland, clovers and lucerne (*Trifolium repens*, *T. pratense*, and *Medicago sativa*) have much higher requirements of calcium and copper for successful growth and nodulation than the tropical species *Desmodium uncinatum* and *Phaseolus lathyroides* (Andrew & Bryan, 1955, 1958; Bryan & Andrew, 1958). The different responses to calcium probably reflect variations in uptake from poor soils rather than in the amount required. Similar effects may explain differing responses to molybdenum by species of *Medicago* and *Trifolium* (Andrew & Milligan, 1954). Successful nodulation in highly acid soils is reported for kudzu (*Pueraria phaseoloides*) in Puerto Rico (Loustalot & Telford, 1948) and for *Acacia mollissima* in Natal (Orchard & Darby, 1956).

Symbiotic nitrogen fixation seems to be sensitive to temperature, but little is known about the behaviour of nodulated tropical legumes in this respect. Meyer & Anderson (1959) grew subterranean clover (*Trifolium subterraneum*) on agar at 20° and 30°C. Plants at both temperatures were well nodulated, but at 30° fixation by inoculated plants was disturbed and they grew poorly. Uninoculated plants grew well with nitrate at both temperatures, suggesting a specific inhibition of fixation at the higher temperature. Similar effects occurred in pot experiments at temperatures above 25°C. In this species high soil temperatures seem to cause inefficient fixation rather than shedding of nodules. Jones & Tisdale (1921) also studied the effect of temperature

obscure. La Flize (1892) recorded excellent growth of barley mixed with peas and vetches, suggesting that it obtained by 'symbiosis' part of the nitrogen fixed by the nodules of the legumes. His data were consistent with this conclusion, but scarcely adequate to prove it. Lyon & Bizzell (1911) stated that cereals and pasture grasses grown with legumes had more protein than if grown alone, and suggested that grasses took up nitrogenous compounds excreted by legumes, or contained in shed roots and nodules. Their published data show an increased protein percentage in the cereals, but do not prove a higher nitrogen content per plant. These authors, apparently unaware of the work of La Flize, called the effect a 'heretofore unnoted benefit from the growth of legumes'. More satisfactory but still hardly conclusive evidence of excretion of nitrogenous compounds from roots of legumes was given by Lipman (1912).

These conclusions were largely ignored, excretion by legumes receiving little attention until the question was re-opened by Virtanen and his colleagues at Helsinki. Virtanen, von Hausen, & Karström (1933) reported substantial excretion of amino-acids by roots of nodulated peas and their utilization by associated non-legumes. Demidenko & Timofeyeva (1937a, b) reported transfer of nitrogen from peas to oats, Lebedev (1949) found a similar transfer from lupins to hemp, and Nowtówna (1937) confirmed these results for several mixed cultures. In natural conditions the North American leguminous tree *Robinia pseudacacia* has a favourable effect, probably due to increased soil nitrogen, on associated plants, e.g. *Catalpa* (McIntyre & Jeffries, 1932), *Fraxinus*, *Liriodendron*, *Quercus*, and *Ulmus* (Chapman, 1935). Jago (1949) recorded similar effects with the trees *Enterolobium cyclocarpum* and *E. saman* in Malaya. Virtanen, von Hausen, & Laine (1937a, b) and Virtanen & Laine (1939) reported that transfer of nitrogen to associated plants could reach a point where the legumes showed signs of nitrogen shortage.

Many workers (Bond, 1938, 1941; Bond & Boyes, 1939; Chapman, 1943; Engel & Roberg, 1938; Ludwig & Allison, 1937; Romashev, 1939; Shapter, 1939; Trumble & Strong, 1937) were, however, unable to detect excretion, which occurred readily and consistently at Helsinki but was often erratic or absent elsewhere. Wilson & Burton (1938), working in Virtanen's laboratory, observed excretion but could not induce it regularly at Madison, Wisconsin. A rather critical balance, sensitive to climatic conditions, between carbohydrate and nitrogen metabolism seems necessary for excretion to occur on a significant

and heat stress. They may thus shed nodules frequently, providing organic matter whose breakdown in the soil would release nitrogen for other plants. Nodules shed (Tschirch, 1887) when the host plant fruits have probably lost much of their nitrogen. Pate (1958a) calculated that less than 3 per cent of the nitrogen fixed in the growing season by *Pisum arvense* is retained in the senescent nodules. The root system has only 6 per cent of the nitrogen in mature plants of *Vicia faba* (Emmerling, 1900).

Butler & Bathurst (1956) calculated that in New Zealand experiments the legume in a white clover - rye grass pasture released 71 lb N/acre/year (81 kg N/ha/year) to the soil in shed nodules. This calculation, though based on several assumptions, probably gives a reasonable estimate of the rate at which nitrogen becomes available in this way in the soil beneath a clover-rich pasture. Transfer by shed nodules is likely to be much more regular than by the excretion of organic nitrogenous compounds, which under most conditions provide only insignificant amounts of nitrogen. Some nitrogen reaches the soil in fallen leaves and stems of clover, but senescent leaves lose much nitrogen to other parts of the plant before falling. Shedding of roots (as distinct from nodules) may, however, release appreciable amounts of nitrogen in the soil. Other workers in New Zealand (Sears, Lambert, & Thurston, 1953; Walker, Orchiston, & Adams, 1954) calculated that in field pasture trials 64 to 80 lb N/acre (72 to 90 kg N/ha) passed from clover to grass. White clover, which transfers more nitrogen than red clover, may transfer 50 per cent of the nitrogen fixed by its nodules. In pasture some nitrogen must pass from clover to grass via grazing animals, which eat protein-rich clover and return part of its nitrogen to the soil in their excreta. Nitrogen in urine is probably directly available to plants, but that in faeces may need preliminary breakdown by bacteria.

Johnstone-Wallace (1937) claimed that, in addition to nitrogen, white clover transfers calcium to associated grasses. This might benefit grasses if it applies to deep-rooted legumes drawing nutrients from levels below those exploited by grass roots. The calcium (and magnesium) content of legumes is higher (Daniel, 1934, 1935) than that of grasses, though the nodules contain less calcium than the aerial parts of the plant (Jensen, 1947; Loneragan, 1959).

Pasture legumes and leguminous crops thus add nitrogen to the soil. Less is known about the contribution of leguminous weeds in cereal crops or of legumes growing in natural habitats. Howard (1906) noted that in most wheat-growing districts of India the wheat crop

half the nodules on wild plants of *Medicago lupulina* (black medick) were ineffective; Purchase, Vincent, & Ward (1951) reported similar results for *M. laciniata* in Australia. Ineffective strains reduce fixation but are unlikely to eliminate it entirely.

#### P. Ecological importance of fixation by nodulated non-legumes

The available evidence suggests significant fixation by wild legumes, though further work is needed to assess their part in the general economy of nitrogen. The importance of fixation by nodulated non-legumes is less clear. They are few in number compared with the legumes, and of little direct economic value, but have considerable ecological importance. The information available will be summarized for the eight genera in which nitrogen fixation is established.

*Alnus* (alder). Crocker & Major (1955) and Crocker & Dickson (1957) studied plant succession and soil development on areas in Alaska uncovered at known dates by retreating glaciers. *Alnus crispa* was one of the first woody plants to appear after the newly hared surface had been colonized by mosses and herbs. After twenty-five to forty years a thicket of *Alnus* was almost continuous, but it was a transient community; after about fifty years seedlings of spruce (*Picea sitchensis*) overtopped the alder and gradually shaded it out. The climax forest developing on these sites consists of spruce and hemlock (*Tsuga heterophylla* and *T. mertensiana*). Abundant leaf-fall from alder is important in building up a new soil. Its contribution of nitrogen is also considerable, the net accumulation in the soil being estimated at 55 lb N/acre/year (62 kg N/ha/year) over a period of fifty years. The nitrogen content of the soil fell after alder disappeared and spruce dominated the community. Species of *Alnus* are widespread in the northern temperate zone and also in the Andes of South America, where nodules are reported in *A. jorullensis* var. *spachii* (Castellanos, 1944). *A. glutinosa* occupied much of the lowland swampy areas of Britain after the last glaciation (5,000 to 7,000 years ago) and also occurred in oak woods on the higher ground (Tansley, 1939).

Nitrogen is transferred from *Alnus glutinosa* to *Picea excelsa* grown with it in pots (Virtanen & Saastamoinen, 1936; Virtanen, 1957). It is not known whether transfer occurs in excreted compounds or in shed roots and nodules; transfer in fallen leaves was eliminated by removing them. One alder provided enough nitrogen for good growth in nitrogen-poor soil by one spruce over eleven years, the plants growing too big for their large wooden tubs.



pollen in bog deposits show that, like *Alnus*, it was prominent in the vegetation of many inland European localities soon after the last glaciation (Fraser & Godwin, 1955; Walker, 1955). The species is consistently nodulated in the field; it fixes nitrogen efficiently in experimental conditions, an ability presumably valuable in the pioneer habitats which it favours.

*Myrica*. The genus has about fifty species, which occur in many temperate and sub-tropical regions. *M. gale*, the bog myrtle, a low shrub dominating extensive areas of bog in Britain, and in northern Europe, Asia, and America, is the most studied species in relation to nitrogen fixation. It is of considerable ecological importance in the vegetation of peat bogs. Nodules are recorded in six other species, mostly from North America. Another species of the same family, *Comptonia peregrina*, is nodulated both in North American forests and in European botanic gardens (Ziegler, 1960). It is abundant in the undergrowth of pine forests on sandy and peaty soils, and may be significant in their nitrogen economy if its nodules are capable of fixation.

*Shepherdia*. The genus is confined to North America, where two of its three species (*S. argentea* and *S. canadensis*) are widespread. Raup (1941) and Moss (1953) refer to the vigorous growth of *Shepherdia* species in poor soils, and in Alaska *S. canadensis* is prominent in the early stages of colonization of glacial debris at very low nitrogen levels (Crocker & Major, 1955).

The number of non-leguminous angiosperms capable of symbiotic nitrogen fixation is comparatively small. The genera where fixation is established have little more than 200 species, about fifty being known to be nodulated. Fixation is proved for only a few species, but root-nodules are reasonable *prima facie* evidence of fixation. These plants are more important in natural vegetation than their number might suggest. *Alnus*, *Myrica*, and *Shepherdia* are pioneers in cool, wet climates where few legumes flourish. *Casuarina* dominates great stretches of tropical coastline and some inland areas of Australia and the Pacific Islands. *Coriaria* is important in several parts of its wide range, particularly in New Zealand, where *Casuarina* is absent and the few native legumes have little ecological significance.

Symbiotic fixation by gymnosperms is hard to evaluate by available information, but is unlikely to equal that by non-leguminous angiosperms. Cycads, widely distributed in warm regions, are rarely abundant. In former eras they were a major group and may have been important

in the nitrogen economy of natural vegetation, but perhaps less so than in agricultural or grazing land losing annually substantial amounts of nitrogen in crops or in the bodies of stock. In undisturbed natural vegetation the loss of nitrogen by leaching, erosion, and denitrification may be comparatively small, most of the element circulating in a closed cycle which returns it to the soil in shed plant organs, and in the bodies and excreta of animals. Nitrogen fixation may, in such conditions of equilibrium, benefit the community as a whole rather than the species which perform it.

Glauber (1656) found it was formed in soils impregnated with excreta of herbivorous animals. He recognized saltpetre as a plant nutrient and envisaged a cycle in which it passed between animal, soil, and plant, and back again to animals eating the plant. Natural crystals of saltpetre appear on old walls sheltered from the rain, and mixtures of crude nitrates are formed in soils rich in decaying organic matter, particularly of animal origin. Nitrates may appear as an efflorescence at the surface of the soil, especially in warm dry climates, where soil water evaporates at the surface, leaving behind dissolved salts. Saltpetre formed in this way was for long exported from Egypt and India to Europe. Nitrates accumulate in old graveyards and other soils impregnated with the decomposition products of animal remains or excreta. The nitrates found on old walls presumably arise from ammonia absorbed by the stones or bricks and originating from the decomposition of protein-rich materials. Siemienowicz (1650), another military engineer, described the occurrence of saltpetre in an artillery textbook, *Artis magnae artilleriae pars prima*. It appeared in dark shady places protected from the rays of the sun and from rain or running water, particularly if they had sheltered domestic animals of any kind. Siemienowicz (1650) strongly recommended old battlefields as sites for prospectors seeking deposits of saltpetre, which arose as a final product of decomposition from the bodies of the slain. He remarked with satisfaction that the Polish army of his day derived its gunpowder from the bodies of enemies killed in earlier wars, and expressed the hope that this economical arrangement would continue in the futuro (*'Posteritas . . . ex resolutis in putridinem cadaveribus salnitrosam colligat materiam, pulveresque nostros fulmineos praeeparabit'*).

Chaptal (1797), summarizing the views of his time on the formation of saltpetre, emphasized that calcareous soils and stones produced in comparable conditions more saltpetre than other sorts. Saltpetre formed in caves was attributed to the percolation of water from overlying soil containing decomposing animal and vegetable remains. Considerable importance was attached to illumination; dim light gave more saltpetre than either darkness or full daylight. Chaptal (1797) described 'nitre beds' or 'nitre plantations' for the artificial production of saltpetre from organic materials. The beds were usually covered to keep out rain and strong sunlight. Materials of plant or animal origin were mixed with a porous calcareous soil. The mass was moistened regularly with water, urine, or the liquid percolating from dunghills; liquid draining from the bed was collected and returned to it. Both

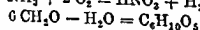
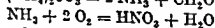
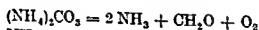
recognition and estimation imply surprisingly good analytical control for the period. Lavoisier, perturbed at this loss in spite of other preoccupations, studied its causes and recommended means for its prevention in one of his last published works. The paper contains much background information and ends with a detailed examination of the problems involved in price-fixing by a monopoly, especially with a product containing variable amounts of unwanted material.

Nitrification thus attracted considerable attention in the early days of industrial chemistry, though it was not clearly recognized as a biological process. Since the early nineteenth century, biological nitrification has been studied mainly in relation to soils; the behaviour in natural waters of nitrate arising from sewage has also received much attention.

Müntz (1887*b*, 1890) found abundant nitrifying bacteria in eroding rocks on peaks over 3,000 m, notably on the Faulhorn, an Alpine peak of rotten calcareous rocks whose whole mass they invaded. These bacteria are active only in the short summer season, low temperatures inhibiting them for the rest of the year, though the cells remain viable through the winter. They are heterotrophic and probably use organic matter carried as dust in the atmosphere and dissolved in rain. Müntz (1887*b*) found traces of ethyl alcohol in rain on the Pic du Midi at about 3,000 m, and showed it to be evolved from soils during the decomposition of organic residues. Traces of ammonium in the atmosphere were assumed to provide the nitrogen supply of the bacteria. Nitrification by organisms receiving carbon and nitrogen only as vapours was demonstrated experimentally. Nitrate was produced in a dish of calcined soil inoculated with a nitrifying organism and enclosed with beakers containing 5 per cent aqueous ethyl alcohol and 1 per cent aqueous ammonium carbonate. Alcohol and ammonium carbonate volatilized from these solutions were the only sources of assimilable carbon and nitrogen available to the organism. Atmospheric sources no doubt provide some organic carbon and combined nitrogen for nitrifying bacteria in the mountains. Their considerable activity suggests, however, the use of other sources, perhaps formed by blue-green algae, found abundantly on rocks at high altitudes by Odintsova (1941) and Krasilnikov (1956).

Müntz (1887*b*, 1890) stressed the part of nitrifying bacteria in the disintegration of rock and its transformation into soil. They also corrode brickwork, forming calcium nitrate (Tolomei, 1894). Pochon, Rose, Tchan, & Augier (1949) described another bacterial disintegration of

way were, however, fruitless. Some observations pointed to a possible reason for this. Warington (1879) found that glucose inhibited nitrification in soil cultures and later (Warington, 1884, 1888) that carbonates were required. Heraeus (1888), finding that nitrifying organisms flourished with ammonium carbonate as the sole source of carbon and nitrogen, suspected that organic matter depressed nitrification. He inoculated nitrifying organisms from soil into two cultures; one contained mineral salts and ammonium carbonate; the other was identical except for the addition of glucose. Nitrite formation was much more active in the sugar-free medium, and the nitrifying bacteria multiplied very rapidly. This was a most startling result, as Heraeus pointed out, for only chlorophyll-containing plants were then known to assimilate carbon dioxide as such or as carbonates. The nitrifying bacteria had no chlorophyll, yet they flourished and multiplied with only inorganic sources of carbon. These results were confirmed by Hueppe (1888) who, in a paper with the fascinating title 'Ueber Chlorophyllwirkung chlorophyllfreier Pflanzen', summarized the changes in a nitrifying culture (presumably mixed) by the following equations:



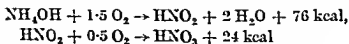
These equations, though not entirely in accord with current ideas, make the fundamental point that nitrifying organisms produce carbohydrate from carbonates. Their formation of organic compounds from carbon dioxide has been confirmed by later workers, e.g. Lozinov & Yermachenko (1957) using *Nitrosomonas europaea*.

Nitrifying organisms were at last isolated in pure culture by Winogradsky (1890). He tried a simple medium containing potassium phosphate, magnesium sulphate, potassium carbonate, and ammonium chloride, with potassium tartrate as the only carbon source. This medium stopped rather than favoured nitrification. Further tests were made with media each lacking one of the original ingredients. Only the medium without tartrate supported nitrification. Organic matter inhibited the autotrophic nitrifiers. Gelatine plates were thus unsuitable for their isolation. The first pure cultures were in liquid media; later (Winogradsky, 1891a, b) solid inorganic media based on silica gel were used. Winogradsky (1891b) isolated in pure culture *Nitrosomonas*, oxidizing ammonia to nitrite, and *Nitrobacter*, oxidizing nitrite to

1890); 33 (Hes, 1937); 70 (Engel, 1929); and for *Nitrobacter*: 76 (Nelson, 1931); 100 (Meyerhof, 1916); 135 (Winogradsky, 1890). The proportion of released energy used for carbohydrate synthesis is clearly low; Baas-Becking & Parks (1927) calculated it as 6.2 per cent for *Nitrosomonas* and 7.5 per cent for *Nitrobacter*. Over 90 per cent of the energy derived from oxidation by *Nitrobacter* (Meyerhof, 1916) and *Nitrosomonas* (Hes, 1937) is dissipated as heat.

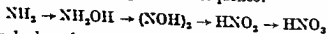
#### D. The biochemistry of nitrification

The conversion of ammonia to nitrate may be written in two stages:



The first reaction is catalysed by *Nitrosomonas*, the second by *Nitrobacter*. Neither metabolizes organic nitrogen compounds, which are only nitrified after ammonia has been split off by heterotrophic micro-organisms (Omeliansky, 1899). The organisms have a small endogenous respiration, as shown for *Nitrobacter* (Bömeke, 1939; Engel, Krech, & Friederichsen, 1954), and for *Nitrosomonas* (Hofman & Lees, 1952; Ruhan & Zavarzin, 1955). The latter authors state that some ammonia is produced catabolically and can be nitrified. The cell-substance of the nitrifying bacteria consists largely of protein, which produces on hydrolysis the usual range of amino-acids (Hofman, 1953; Engel *et al.*, 1954). Silver (1960) found that *Nitrobacter* used formate, but not acetate, citrate, lactate, or glucose.

The stages between ammonia and nitrate remain somewhat obscure. Two intermediates in addition to nitrite are required if the oxidation proceeds in two-electron steps. Mumford (1914) and Corbet (1935) reported hydroxylamine and hyponitrite as intermediates in bacterial oxidation of ammonium. Since their cultures were probably mixed, it remains uncertain whether these compounds arose in nitrification. Kluyver & Donker (1926) proposed the sequence:



Here again hydroxylamine and hyponitrite (or one of its isomers) appear; they are, indeed, hard to avoid in writing schemes of this nature. Hydroxylamine is toxic to *Nitrosomonas*, as to all other organisms, except at very low concentrations. Direct study of its metabolic rôle is thus difficult. Meyerhof (1917) noted the disappearance of added hydroxylamine in cultures of *Nitrosomonas*. He did not con-

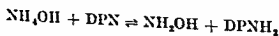
obscure, largely because of difficulties in growing the organisms in pure culture on a scale yielding enough material for metabolic study.

### E Heterotrophic nitrification

The nitrifying organisms so far considered are autotrophic. Nitrification is, indeed, often stated to be strictly associated with autotrophy. There are, however, many published reports of nitrification by heterotrophic organisms. Early statements to this effect were severely criticized by Winogradsky (1904), and more recent writers, e.g. Bömeke (1930), have been equally sceptical of later work. Many supposed cases of heterotrophic nitrification may be due to traces of nitrite and nitrate in reagents, and of nitrogen oxides in laboratory air. These obvious sources of error appear, however, to be adequately controlled in some modern work.

*Aspergillus flavus* produces nitrate and nitrite from peptone in pure culture (Schmidt, 1954, Iyengar & Hora, 1959). The latter authors found that a *Penicillium* oxidized nitrite to nitrate, but did not form nitrite from peptone. Fisher, Fisher, & Appleman (1952, 1956) isolated from soil several heterotrophic bacteria which under carefully controlled conditions oxidized ammonia to nitrate. Oxidation of ammonia is unlikely to be important in the energy balance of these species. They seem, however, to be abundant in the soil, where their total nitrification may be significant. Some earlier workers, e.g. Cutler & Mukerji (1931), also reported slight nitrite formation from ammonia by heterotrophic soil bacteria in experiments without obvious sources of error. These heterotrophs nitrify more completely than the autotrophic species. *Aspergillus flavus* forms ammonia from peptone, a step of which autotrophic nitrifiers are incapable, before oxidizing it to nitrite and nitrate. No autotroph is known to perform both oxidations. *Streptomyces nitrificans*, which obtains its carbon, nitrogen, and energy requirements from urethane, forms some nitrite from this compound, nitrate does not appear. It also nitrifies urea and ammonium carbonate (Schatz, Isenberg, Angrist & Schatz, 1954; Schatz & Mohan, 1955).

Klausmeier & Bard (1954) reported *Bacillus subtilis* to contain an enzyme catalysing the reversible oxidation of ammonia to hydroxylamine according to the equation



Roussos, Takahashi & Nason (1957) confirmed production of ammonia from hydroxylamine by an enzyme from this organism, but attributed

large amounts of sulphate in seedlings of *Lupinus luteus* and of nitrate in those of *Cucurbita pepa*, concluded that these oxidized compounds arose from protein during termination. Later (Belzung, 1893) he withdrew this suggestion with regard to nitrate, whose accumulation in seedlings he attributed to very efficient absorption of traces in the medium, sulphate was still held to be formed by oxidation of protein sulphur. Bach (1913) claimed that nitrite arose by enzymatic oxidation in sterile potato juice. None was formed in the absence of oxygen, a little appeared in juice heated to boiling. Maze (1911b, c) recorded a similar production of nitrite in sterile juice from etiolated pea seedlings. He also found nitrite in maize seedlings cultivated with ammonium as the sole source of nitrogen (Maze, 1912). Later (Maze, 1915), he studied nitrite formation in seedlings of pea (*Pisum sativum*), maize (*Zea mays*), and vetch (*Vicia narbonnensis*) grown in sterile culture without nitrate. At 30°C a little nitrite appeared transiently, at the extreme temperature of 56°C it occurred in larger amounts and for longer periods. Both oxidation of ammonia and reduction of nitrate were held to occur in the seedlings, the former being the more accelerated by rising temperature. Ammonia was also oxidized in distilled water at 56°C, and in the seedlings may have formed nitrite non enzymatically.

Malavolta (1954) found nitrate in seedlings of rice (*Oryza sativa*) whose only nitrogen source was ammonia and suggested that it was formed by an oxidative detoxification mechanism. Nitrate has also been reported in seedlings of tomato (Clark, 1936) and barley (McKee, 1950) supplied with ammonia, but nitrification in the nutrient solution was not excluded, as it is stated to have been in Malavolta's work.

Khudairi (1957) found 'large amounts of nitrates, the presence of which is attributed to fixation of the atmospheric nitrogen by bacteria' in root nodules of *Prosopis stephaniana*, a leguminous shrub abundant in Iraq. The actual amount of nitrate is not stated, nor the method used to detect it. Cheniae & Evans (1957) found an adaptive nitrate reductase in soybean nodules. Nitrate induced the enzyme in cultured rhizobia, but not in nodules of intact plants, suggesting that external nitrate did not enter them. The presence of the adaptive enzyme and of nitrate in nodules thus suggests oxidation of ammonia synthesized by rhizobia.

Increases of nitrate in detached leaves are recorded for several species: tobacco (*Nicotiana tabacum*) (Vickery, Pucher, Wakeman, & Leavenworth, 1937), buckwheat (*Fagopyrum esculentum*), sorrel (*Rumex acetosa*) and wheat (*Triticum sativum*) (Moyse, 1949, 1950),



## CHAPTER 5

# DENITRIFICATION

### A. General

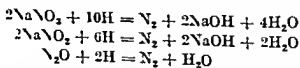
The term denitrification is applied to biological decompositions, in the soil and elsewhere, in which nitrogen is liberated in gaseous form. The main gaseous product is molecular nitrogen,  $N_2$ , nitrous oxide,  $N_2O$ , is also formed and in some cases nitric oxide,  $NO$ . These processes may cause considerable losses of nitrogen from the soil. In New Zealand pasture soils Walker, Adams, & Orchiston (1956) found that about one third of the nitrogen added in fertilizers was lost, 'almost certainly by denitrification'. Similar losses of nitrogen, established by methods using  $N^{15}$ , are reported by MacVicar, Garman, & Wall (1950) and by Jones (1951). Arnold (1954) recorded losses of nitrous oxide from the soil. De & Dagar (1954, 1955) found with water logged rice soils in India that 26 to 31 per cent of the nitrogen added in plant residues was lost as gas, losses from ammonium sulphate were 31 to 34 per cent and from sodium nitrate 44 to 45 per cent. These figures are for soils without any crop, losses from soil under a rice crop were smaller but still substantial. Considerable losses of nitrate from water logged soils in England (Lawes, Gilbert, & Warington, 1881) were attributed to production of molecular nitrogen. Serious losses of nitrogen from heavily manured soils thus occur in both temperate and tropical conditions. In most unfertilized soils competition by plant roots and other micro organisms for the small amounts of available nitrogen may, however, reduce the activity of denitrifying species to a comparatively low level.

Reiset (1856, 1889), following the changes in nitrogenous compounds during the decomposition of meat and the maturation of animal manure, concluded that these processes were accompanied by losses, sometimes considerable of gaseous nitrogen. Smith (1867), noting a rapid decrease of nitrate in rivers where it was formed from sewage, suggested that it was probably decomposed to nitrogen gas. Reiset (1868) showed that nitrous oxide was evolved in fermentation of sugar beet juice. He attributed this to an oxidation of ammonia in the juice instead of the reduction 'as stated commonly' of nitrate. Schloeding (1869) showed that in tobacco juice allowed to putrify the

(Allen & Van Niel, 1902) is *Pseudomonas stutzeri* (Lehmann et Neumann) Kluyver. It released nitrate nitrogen largely as gas but used 20 per cent in the synthesis of organic matter. Breal (1892) got similar results with an organism isolated from straw. The ability to reduce nitrate to nitrite was shown to be common among bacteria by several early workers e.g. Frankland (1888) and Warrington (1888). Fewer organisms reduce nitrite further to nitrogen but they are common in nature. Another denitrifying bacillus was isolated from horse-dung by Schirokich (1896); similar organisms are widespread in soils and waters, including the sea (Baur, 1902; Parlandt, 1911; Lloyd, 1931; Sreenivasan & Venkataraman, 1956a, b).

## B Metabolic relations of denitrification

Gayon & Dupetit (1882a), Deberain & Maquenne (1883), and Munro (1886) noted the need for fermentable organic matter in anaerobic denitrification. Giltay & Aberson (1892) and Weissenberg (1897) treated denitrification as equivalent to aerobic respiration, the oxygen of nitrate replacing that of the air in the energy producing oxidation of carbohydrate. Denitrification is quite distinct from assimilation of nitrogen. Some denitrifying bacteria are unable (Rusakova & Butkevich, 1941; Baalsrud & Baalsrud, 1954) to assimilate nitrogen from nitrate, others (Marshall, Disliherger, MacVicar, & Hallmark, 1953) use it much less efficiently than ammonia. Most denitrifying species seem unable to reduce nitrate to the more readily available form of ammonia. Modern views of denitrification emphasize the supply of hydrogen for reduction as in the equations

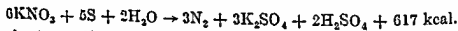


Organic compounds in great variety, particularly organic acids, sugars and alcohols (serve as hydrogen donors (or more correctly as electron donors) in different denitrifying bacteria. Inorganic substances such as molecular hydrogen, thiosulphate, sulphur or hydrogen sulphide are effective electron donors for some species. The replacement of atmospheric oxygen by the oxygen of nitrate in oxidizing respiratory substrates suggests immediately that denitrification is essentially an anaerobic process. The precise extent to which it is inhibited by oxygen is uncertain mainly because of difficulties in estimating the oxygen available to bacteria in cultures aerated to varying degrees. Sacks &

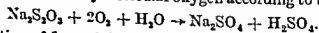
heterotrophic denitrifiers can grow anaerobically without nitrate, fermenting glucose to lactic acid, glycerol, and 2,3-butanediol.

*Micrococcus denitrificans*, thoroughly studied by Kluyver & Verhoeven (1954a, b), appears to be largely autotrophic, using molecular hydrogen instead of organic electron donors in the reduction of nitrate to nitrous oxide and molecular nitrogen. It is not, however, completely autotrophic, being unable to synthesize certain organic metabolites required only in small amounts but essential for its growth. They can be supplied by addition of a small amount of yeast autolysate to the culture medium. This organism can switch from molecular oxygen to nitrate as the basis of its metabolic activities, both added substrates and cellular reserve materials being respired equally effectively with either source of oxygen. It shows equal versatility in passing from hydrogen to organic substrates. The enzyme systems necessary for reduction of nitrate and for activation of hydrogen are both adaptive; the latter is quite independent of the dehydrogenases acting on organic substrates.

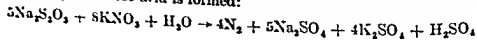
*Thiobacillus denitrificans* (first isolated by Beijerinck, 1904) is of considerable interest through its ability to use elemental sulphur as an electron donor in the reduction of nitrate. The overall equation for the process may be written:



Liesko (1912) showed the species to be an obligate chemolithotroph, unable to metabolize organic substances. It has more recently been investigated by Baalsrud & Baalsrud (1954), who grew it in a purely inorganic medium containing nitrate and thiosulphate. Nitrate can be replaced by nitrite, nitrous oxide, or nitric oxide, and thiosulphate by sulphur, hydrogen sulphide, or sodium dithionate ( $\text{Na}_2\text{S}_4\text{O}_6$ ). Thio-sulphate is also oxidized by molecular oxygen according to the equation:

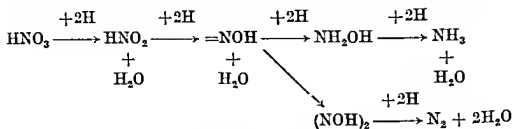


The production of free sulphuric acid in oxidations using both nitrate and molecular oxygen makes its neutralization essential for continued activity of the organism, which lacks the remarkable resistance to acidity of *T. thio-oxidans*, its optimum reaction is pH 7. Both in the oxidation of thiosulphate by atmospheric oxygen, and in that of sulphur by nitrate, a substantial part of the sulphur metabolized appears as free sulphuric acid. When thiosulphate is oxidized by nitrate, comparatively little free acid is formed:



was suggested, but the evidence for it was not entirely conclusive. The enzyme preparations contained firmly bound cytochrome c.

Several schemes have been put forward for the sequence of intermediates in denitrification. The most plausible is probably that of Kluyver & Verhoeven (1954a), which may be summarized as follows:



This scheme meets the condition, usually assumed for biological oxido reductions, that changes of oxidation state occur by two electron steps. It also has the merit of being clear and easily understood. It is, however, in part highly hypothetical. There is general agreement that the first step is the reduction of nitrate to nitrite. The reduction product of nitrite is uncertain, Kluyver & Verhoeven suggest the free radical nitroxyl ( $=\text{NOH}$ ). The postulated reactions follow the familiar course of hydrogen transfer from a donor via a carrier molecule to an acceptor, but the reacting molecules are not firmly identified. The hypothetical nitroxyl has a most strategic position in the sequence, commanding two alternative pathways. One leads by two more two-electron reductions to hydroxylamine and ammonia, the other, of more immediate interest for denitrification, leads to a dimer  $(\text{NOH})_2$  which takes up two more hydrogen atoms to yield molecular nitrogen and water. The pathway to hydroxylamine and ammonia is blocked in organisms (e.g. *Thiobacillus denitrificans*) that denitrify but cannot assimilate nitrate.

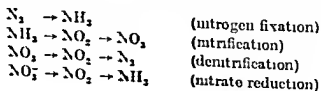
A molecule containing two nitrogen atoms must be formed in passing from nitrate or nitrite, with one nitrogen atom per molecule, to nitrous oxide and nitrogen gas, with two nitrogen atoms per molecule. Dimerization of nitroxyl is very plausible. Several dimers are possible in theory. Hyponitrous acid ( $\text{H}_2\text{N}_2\text{O}_2$ ) is one but may not be an intermediate as attempts to denitrify it using *Pseudomonas aeruginosa* and *Micrococcus denitrificans* (Kluyver & Verhoeven 1954a) and *P. stutzeri* (Allen & Van Niel 1952) were unsuccessful. The position with its isomers is not clear. Allen & Van Niel (1952) claimed that *P. stutzeri* hydrogenated nitramide ( $\text{H}_2\text{N}-\text{NO}_2$ ) yielding molecular nitrogen. This was not confirmed by Kluyver & Verhoeven (1954a), who considered nitramide too unstable to test as a substrate and suggested

of nitrite by ascorbic acid or DPNH is slow at pH 6, but accelerated by increasing acidity (Evans & McAuliffe, 1956). Nitrite is also reduced by a derivative of *p* hydroxycinnamic acid (Taborsky, Cammarata, & Fruton, 1957, Zioudrou & Fruton, 1957, Zioudrou, Meyer, & Fruton, 1957), which is oxidized to the corresponding derivative of *p* hydroxy mandelic acid. Zioudrou and her co workers suggest that nitrite may take part in the biological oxidation of isoeugenol to dehydrodihydroeugenol. The reaction, which occurs readily *in vitro* at pH 6, is of interest since these phenylpropene derivatives may be precursors of lignin.

These or similar reactions may take part in some biological denitrifications. Quantitative studies of denitrification by several bacteria (Van Olden, 1940, Sacks & Barker, 1952, Allen & Van Niel, 1952, Kluyver & Verhoeven, 1954*a, b*) show that nitrogen equivalent to the nitrate or nitrite consumed appears in gaseous products. A reaction of the Van Slyke type involving amino groups would evolve twice the amount of nitrogen of the original nitrate or nitrite. Iwasaki, Matsubayashi, & Mori (1956) found evidence for such a reaction with an unidentified soil bacterium. With *p* phenylenediamine or lactate as hydrogen donor it gave off as gas twice the nitrogen supplied as nitrite. A reaction with amino groups is plausible here, but seems unusual. The amino groups of phenylenediamine might react with nitrite, but endogenous amino groups must have been involved in cultures supplied with lactate. Buchner & Rapp (1901) noted that yeast press juice produced nitrogen gas from added nitrite. They attributed this to a purely chemical reaction between nitrite and amino acids in the juice.

#### F. General considerations on the metabolism of inorganic nitrogen

We have now considered the main transformations of inorganic nitrogen compounds induced by organisms. The reactions are mainly microbiological. Higher plants reduce nitrate to ammonia, their ability to nitrify ammonia or to denitrify nitrate remains doubtful, and at most is small, fixation of gaseous nitrogen seems to require free living or symbiotic micro organisms. The biochemistry of these processes is established only in broad outline. The following partial sequences are clear



## CHAPTER 6

# ASSIMILATION OF ORGANIC NITROGENOUS COMPOUNDS

### A. Urea and Urcides

Urea was probably the first organic compound to be studied as a source of nitrogen for higher plants; it is also the main organic substance applied individually in present agricultural practice as a nitrogenous manure, though dung and other organic fertilizers contain various nitrogenous compounds. Cameron (1858) recorded in a brief abstract elaborate experiments on assimilation of urea by barley growing in soils and atmospheres freed from nitrogenous compounds. He concluded that urea, absorbed without conversion to ammonia, was an effective source of nitrogen. Similar results were reported by Ville (1862, 1863) and by Hampe (1865, 1868). These early studies did not exclude the possibility of bacterial transformation of urea before entry into the plant. Lutz (1898) and Hansteen (1899) showed that plants in sterile culture also absorbed urea. Hansteen (1897) found that the minute aquatic angiosperm *Lemna* used urea, asparagine, or ammonia, but not nitrate, for protein synthesis in the dark. Yamaguchi (1930) and Tanaka (1931) showed by microchemical tests with xanthidrol that in *Sisyrinchium bermudianum*, *Brassica chinensis*, *Plantago major*, and *Zea mays* urea entered the roots unchanged.

Rapid uptake of urea, usually followed by good growth in plants using it as the sole source of nitrogen, was reported by Suzuki (1897) (seedlings of wheat and *Lupinus luteus*; detached shoots of potato and of *Halesia hispida*, Styracaceae), Thomson (1899) (oats and barley), Chick (1903) (*Chlorella pyrenoidosa*), Hutchinson & Miller (1912) (peas), Beaumont, Larninos, Pickenbrock, & Nelson (1931) (tobacco), Loo (1946) (*Baeria chrysostoma*, Compositae), Reifer & Melville (1949) (rye-grass), and Newton (1957) (wheat). Excised roots of peas (Goas, 1959) and of *Pinus serotina* (Barnes & Naylor, 1959) use urea as the sole source of nitrogen. Many species thus absorb urea through the roots; it is also, as will be seen later in this section, assimilated through the leaves. Roach (1939) reported good utilization of urea injected into trunks of apple trees.

unavailable for radish seedlings (Molhard, 1909b) Lutz (1898) reported toxicity for allylamine, benzylamine, diphenylamine, aniline, naphthylamine, pyridine, piperidine, and several alkaloids (atropine, caffeine, cocaine, morphine, quinine) Caffeine and theobromine were also toxic to radish seedlings (Molhard, 1911a) The algae *Ulothrix subtilis* and *Spirogyra crassa* obtained nitrogen from atropine and morphine, but not from quinine or strychnine (Comere, 1919) Virtanen & Schwyzer (1951) showed that peas in sterile culture assimilated dimethylamine, trimethylamine, ethylamine, propylamine, and isopropylamine, the greatest uptake was with ethylamine

### C Amino-acids

Several amino acids are effective sources of nitrogen for some green plants in sterile and other cultures Species differ considerably in their ability to use individual amino acids Wolf (1868) found that rye grew well in water culture with tyrosine as the sole source of nitrogen Wagner (1869), Schreiner & Reed (1908), and Molhard (1909a, 1919) showed that the nitrogen of glycine was available for various higher plants, as found for wheat in sterile culture by Newton (1957), alanine was also used by several species though it was toxic to radish seedlings (Molhard, 1909a) For most species neither glycine nor alanine equalled nitrate as a source of nitrogen Schreiner & Skinner (1912) reported that arginine, histidine, creatine, or creatinine could replace nitrate for wheat seedlings, they considered creatine a normal constituent of soils It occurs in animal tissues and urines, and may reach the soil from this source A soil bacterium (*Pseudomonas ovalis*) breaks down creatine to sarcosine and urea (Appleyard & Woods, 1956)

Ghosh & Burris (1950) found alanine, asparagine, histidine, and phenylalanine better single sources of nitrogen for clover (*Trifolium pratense*) than either ammonium salts or nitrates, several other amino acids were also utilized Tomato plants used a wide range of amino acids, several were better nitrogen sources than ammonium, but only glutamic acid was better than nitrate Ratner, Kolosov, Ukhina, Dobrokhotova, & Kazuto (1956) studied the utilization of amino acids by maize (*Zea mays*) and sunflower (*Helianthus annuus*) in sterile culture Arginine, aspartic acid, glutamic acid and glycine were effective nitrogen sources though inferior to inorganic nitrogen Alanine and lysine were poor sources of nitrogen, phenylalanine and tyrosine inhibited growth Tyrosine labelled in the carboxyl group with  $C^{14}$  was taken up as the intact molecule Carbon from labelled glycine

and to interactions between individual compounds Brown (1906) found asparagine the best of several nitrogen sources for isolated barley embryos. It alone produced growth of the root system, growth of the shoot occurred also with ammonium sulphate, aspartic acid, glutamic acid, and potassium nitrate. Leucine, phenylalanine, and tyrosine inhibited growth. More recent workers have confirmed the inhibitory effect of single amino acids, e.g. Spoerl (1948) with orchid embryos, and Stokes (1953) in embryos of *Heracleum sphondylium*. In each case arginine was the only amino acid giving good growth as the sole source of nitrogen. Rijken (1955, 1956) found the glutamine supply to control growth of isolated embryos of *Capsella bursa pastoris*, they grew, but only slowly, when glutamine was replaced by a mixture of seventeen other amino acids. Glutamic acid was not a good source of nitrogen. Asparagine inhibited growth, possibly by competition with glutamine, except at low concentrations (10 mg/l or below). Asparagine at somewhat higher concentrations also inhibited young embryos of *Arabidopsis thaliana* and of *Reseda odorata*. Asparagine at 400 mg/l stimulated growth in embryos of all other species tested (*Allium cepa*, *Anagallis ariensis*, *Chenopodium album*, *Cleome viscosa*, *Datura stramonium*, *Hordeum sativum*, *Medicago orbicularis*, *M. tribuloides*, and *Sisymbrium orientale*). Embryos of all these species, however, grew better with glutamine than with asparagine, even though they used both amides. Glutamine is more effective than asparagine as a precursor for protein synthesis in older seedlings. Kretovich & Yevstigneyeva (1953) infiltrated solutions of both amides into nitrogen starved wheat seedlings (16 days old) and observed distinctly greater formation of protein with glutamine than with asparagine. Ammonium glutamate was also more favourable to protein synthesis than ammonium aspartate.

Harris (1956) showed that isolated embryos of oats (*Avena sativa*) made good growth with casein hydrolysate as their source of nitrogen. A mixture of 18 amino acids was also effective, most of these amino acids, however, inhibited growth if supplied singly. Similar interactions between individual amino acids occur in various other plant organs, e.g. prothallia of *Gymnogramme calomelanos* (Sossountzov, 1950a, b, 1952), pea seedlings (Fries 1951), tobacco seedlings (Pratesi & Ciferri 1946), embryos of *Datura* (Sanders & Burkholder, 1948), and isolated roots of *Senecio vulgaris* (Skinner & Street 1954).

Several workers have studied the role of amino acids in the nutrition of green micro-organisms. Braarud & Føyn (1931) showed that a species



The antibiotic griseofulvin, produced in the soil by several mould fungi, is another fairly large organic molecule absorbed by plant roots. It is taken up by roots of lettuce (*Lactuca sativa*) and translocated to the leaves, from which it is excreted in watery exudations (Brian, Wright, Stubbs, & Way, 1951). Krasilnikov (1951) showed that clover, maize, pea, and wheat plants took up aureomycin, streptomycin, and penicillin through the roots, the antibiotics were detected in stems and leaves. Aureomycin is also absorbed by roots of *Phaseolus lunatus* (Blanchard & Diller, 1951). Many soils, especially those rich in organic matter, must contain metabolites of micro-organisms in considerable variety, though normally in very low concentrations. Kolosov & Ukhina (1954) reported that in the roots of maize plants grown in sterile culture, synthesis of amino-acids, particularly alanine, glutamic acid, and serine, was stimulated by metabolic products of soil micro-organisms. The material added contained only traces of amino acids. In this work, as in various other studies, e.g. Kursanov, Tuyeva, & Vereshchagin (1954), Kursanov (1955), Turchin, Gumin'skaya, & Plyshovskaya (1955), Yemm & Willis (1956), the roots were a major site of amino acid synthesis.

Free amino acids in the soil could arise by the breakdown of protein containing organic residues. Numerous species, mostly legumes, are known to excrete small amounts of amino acids through the roots (Kandler, 1951, Frank, 1954, Butler & Bathurst, 1956, Dehay & Care, 1957, 1958, Rovira, 1956, 1959). Katznelson, Rouatt, & Payne (1945) showed with seedlings of several species that drying and subsequent moistening of the roots markedly increased excretion of amino acids. Even if amino acids are continuously excreted, they are more likely to be absorbed by micro-organisms or by plant roots than to accumulate in the soil.

Pastures present a special case where organic nitrogenous compounds reach the soil in comparatively large amounts. Grazing animals return to the soil up to 500 lb organic N/acre/year (560 kg organic N/ha/year) (Raiser & Melville, 1949). Over half of this is urea, there are also appreciable amounts of uric acid, both compounds being assimilated by some plants at least. Amino-acids also occur in urine in small amounts. Pasture plants may thus obtain organic nitrogenous compounds in unusual variety and amount. In other types of vegetation the scattered and irregular additions of such compounds in animal excreta may have little general significance. Ability to assimilate urea and uric acid may be advantageous to algae such as *Chlorella pyrenoidosa* (Chick, 1903), which inhabit sewage polluted waters.

*Thamnosma montana* (Rutaceae), *Prosopis juliflora* (Leguminosae), *Sarcobatus vermiculatus* (Chenopodiaceae), and *Viguiera reticulata* (Compositae). Later work (Muller, 1953, Muller & Muller, 1956) confirmed the presence of water soluble toxins in *Encelia farinosa* and in *Thamnosma montana*, inhibiting in laboratory trials the growth of smaller plants frequently found under desert shrubs, e.g. *Cryptantha micrantha* (Boraginaceae), *Chaenactis fremontii* (Compositae), and *Malacothrix californica* var. *glabrata* (Compositae). Extracts of *Franseria dumosa* (Compositae), a shrub consistently sheltering numerous smaller plants, were, however, still more toxic than those of *Encelia farinosa*. The toxins, though effective inhibitors in laboratory tests, seem not to affect seedlings in field conditions. They may be destroyed in the soil by micro-organisms, adsorbed to soil colloids, or leached from the surface layers of the soil by the heavy rain that usually precedes the germination of desert annuals.

Deleuil (1950, 1951a) noted the almost complete absence of annuals in healthy associations containing *Erica multiflora*, *Helianthemum larandulaefolium*, and *Rosmarinus officinalis*. Soil from such areas and its aqueous extract were toxic to seedlings of annuals, soil extracted with water was not toxic. Similar effects were recorded for *Helianthemum nummularium* (Bournérias, 1909). Most annual legumes were sensitive to the toxin, but a few species, e.g. *Errum gracile*, *Hippocrepis ciliata*, and *H. unisiliquosa*, were resistant (Deleuil, 1951b). These species were well nodulated and extracts of their nodules appeared to protect sensitive species against the toxin. The herb *Hieracium pilosella* made soil toxic to seedlings of *Lathyrus aphaca*, *Raphanus sativus*, and other species (Becker, Guyot, Massenet, & Montegut, 1950). Soil in which it had grown was toxic to its own seedlings (Becker, Guyot & Montegut, 1951). Campbell (1959) found in roots and other organs of chow chow (a variety of *Brassica oleracea*) a substance strongly inhibiting the germination of clover (*Trifolium repens*). It had no effect on the germination of species of *Lolium* but markedly reduced the growth of their roots. Guyot (1959) concluded from a study of 111 species in 34 families that there is a positive correlation between the content of soluble solids in the aerial parts and the elimination of phytotoxic substances through the roots. *Helleborus foetidus* (Ranunculaceae) had the highest soluble solids content of the species tested, water in which its roots had been washed completely inhibited germination of 15 species. The soil thus contains soluble organic substances useful or harmful to individual plant species. These substances probably

Skodvin, 1948, Fisher & Cook, 1950, Fisher, 1952, Rodney, 1952), it was also shown that urea enters the leaf through the cuticle as well as via the stomata. Reeves (1954) showed that wheat used nitrogen supplied in urea sprays for protein synthesis. Potato, celery (*Apium graveolens*), tomato, cucumber, maize, coffee, cocoa (*Theobroma cacao*), and banana (*Musa*) absorb urea rapidly through the leaves (Hinsvark, Wittwer, & Tukey, 1953, Cam, 1956, Malavolta, Arzolla & Haag, 1957, Freiberg & Payne, 1957). In several species the absorbed urea appears to be hydrolysed by urease in mature leaves. In banana, however, urease occurs only in actively growing tissues, to which urea is translocated before hydrolysis (Freiberg & Payne, 1957). Inorganic compounds of nitrogen are generally less suitable than urea for foliar application since they tend to damage the leaves. Petinov & Pavlov (1955), however, increased the protein content of wheat grain by spraying the plants at the milk ripe stage with a 3 per cent solution of ammonium nitrate.

#### H. Absorption of Nitrogenous substances by the Leaves of Carnivorous Plants

The specialized organs by which carnivorous plants trap and digest insects and other small animals are modified leaves, with the possible exception of the bladder traps of *Utricularia*. Their morphological specialization is accompanied by unusual metabolic features, particularly in relation to the uptake of complex nitrogenous substances.

The metabolic importance of an extra supply of nitrogen to carnivorous plants has long been recognized. Burnett (1829) wrote of the pitchers of *Sarracenia*: "The water in these receptacles, impregnated by the half decomposing animal matter, doubtless affords a highly nutritive and invigorating diet to the plant, for it is well known that the drainings of dunghills give a powerful stimulus to vegetables, as the rainwater that percolates thro' through dissolves and carries with it, in solution, much of the nutritious and more subtle ingredients of manure, and as the food of plants is chiefly, if not wholly, absorbed in a fluid state, the more soluble manures are ever the best conducive to their growth. Nor must the nitrogen thus afforded to the prehensile plants be overlooked in the account, when we know how potent an excitant ammonia is to the vegetable frame." Burnett also drew attention to the observation of Rumphius (1747) that although most small animals trapped in the pitchers of *Nepenthes* are digested "a certain small squilla or shrimp lives there", he commented that "even this simple digestive apparatus is not free from intestinal worms". This "squilla" must share the re-

*cularis*, the pitcher plant of Western Australia, a taxonomically isolated species which constitutes the family Cephalotaceae; he considered that bacteria were also important in digestion. Morren (1875a) held bacteria responsible for digestion in *Pinguicula vulgaris*, but Dernby (1917) found a proteolytic enzyme in leaves of this species. Reports on *Utricularia* are also contradictory. Adova (1924) considered digestion in the bladders to be enzymatic; Kiesel (1924a) stated that it was bacterial.

TABLE 4

*Amino-acids regularly found in protein*

<i>Common name</i>	<i>Chemical name and structure</i>	<i>References for isolation and recognition as protein constituents</i>
Glycine	$\alpha$ Aminoacetic acid $\text{NH}_2\text{CH}_2\text{COOH}$	Braconnot (1820)
Alanine	$\alpha$ Aminopropionic acid $\begin{array}{c} \text{CH}_3 \\   \\ \text{NH}_2\text{CH}\text{COOH} \end{array}$	Schutzenberger & Bourgeois (1875), Weyl (1888), (synthesized by Strecker, 1850)
Valine	$\alpha$ Aminoisovaleric acid $\begin{array}{c} \text{CH}_3 \text{ CH}_3 \\ \diagdown \quad / \\ \text{CH} \\   \\ \text{NH}_2\text{CH}\text{COOH} \end{array}$	Gorup Besanez (1856), Schützenberger (1879), Fischer (1906b)
Leucine	$\alpha$ Aminoisocaproic acid $\begin{array}{c} \text{CH}_3 \text{ CH}_3 \\ \diagdown \quad / \\ \text{CH} \\   \\ \text{CH}_2 \\   \\ \text{NH}_2\text{CH}\text{COOH} \end{array}$	Proust (1819), Braconnot (1820)
Isoleucine	$\alpha$ Amino $\beta$ methylvaleric acid $\begin{array}{c} \text{CH}_3 \text{ C}_2\text{H}_5 \\ \diagdown \quad / \\ \text{CH} \\   \\ \text{NH}_2\text{CH}\text{COOH} \end{array}$	Ehrlich (1904)
Serine	$\alpha$ Amino $\beta$ hydroxypropionic acid $\begin{array}{c} \text{CH}_2\text{OH} \\   \\ \text{NH}_2\text{CH}\text{COOH} \end{array}$	Cramer (1865)
Threonine	$\alpha$ Amino $\beta$ hydroxybutyric acid $\begin{array}{c} \text{CH}_3 \\   \\ \text{HCOH} \\   \\ \text{NH}_2\text{CH}\text{COOH} \end{array}$	McCoy, Meyer, & Rose (1935)

TABLE 4 (Continued)

*Amino acids regularly found in protein*

Common name	Chemical name and structure	References for isolation and recognition as protein constituents
Hydroxyproline	<p>4 Hydroxypyrrolidine 2 carboxylic acid</p> $  \begin{array}{c}  \text{HOHC} - \text{CH}_2 \\    \quad \quad   \\  \text{H}_2\text{C} \quad \quad \text{CH.COOH} \\  \diagdown \quad \diagup \\  \text{NH}  \end{array}  $	Fischer (1902a)
Aspartic acid	<p><math>\alpha</math> Aminosuccinic acid</p> $  \begin{array}{c}  \text{COOH} \\    \\  \text{CH}_2 \\    \\  \text{NH}_2\text{CH COOH}  \end{array}  $	Plisson (1827), Pasteur (1852), Ritthausen (1868)
Asparagine	<p><math>\beta</math> Amide of aspartic acid</p> $  \begin{array}{c}  \text{CONH}_2 \\    \\  \text{CH}_2 \\    \\  \text{NH}_2\text{CH COOH}  \end{array}  $	Delaville (1802), Vauquelin & Robiquet (1806), Damodaran (1932)
Glutamic acid	<p><math>\alpha</math> Aminoglutaric acid</p> $  \begin{array}{c}  \text{COOH} \\    \\  \text{CH}_2 \\    \\  \text{CH}_2 \\    \\  \text{NH}_2\text{CH.COOH}  \end{array}  $	Ritthausen (1866), Scheibler (1869b), Gorup Besanez (1877)
Glutamine	<p><math>\gamma</math> Amide of glutamic acid</p> $  \begin{array}{c}  \text{CONH}_2 \\    \\  \text{CH}_2 \\    \\  \text{CH}_2 \\    \\  \text{NH}_2\text{CH COOH}  \end{array}  $	Schulze & Barbieri (1877), Damodaran, Jaaback, & Chibnall (1932)
Lysine	<p><math>\alpha, \epsilon</math> Diaminocaproic acid</p> $  \begin{array}{c}  \text{CH}_2 - \text{CH}_2 - \text{CH}_2 - \text{NH}_2 \\    \\  \text{CH}_2 \\    \\  \text{NH}_2\text{CH.COOH}  \end{array}  $	Dreschel (1889)

term is unnecessary and misleading, several amino acids of the D series having been isolated from natural products. D glutamic acid has been reported (Kögl & Erxleben, 1939) in the proteins of animal tumours. This claim has led to much controversy, as small amounts of D glutamic acid can arise from the L acid by racemization during acid hydrolysis, but the possibility that D amino acids are present in some proteins cannot yet be excluded. Their production by micro organisms is well established. *Bacillus anthracis* forms polypeptides of molecular weights up to 50,000 which on hydrolysis yield only D glutamic acid (Bruckner & Ivanovics, 1937), the amino acid residues are linked mainly through  $\gamma$  glutamyl bonds (Bruckner, Kovacs, & Denes, 1953). Various D amino acids occur in antibiotics, e.g. D phenylalanine in gramicidin S (Synge, 1945b), D-dimethylcysteine in penicillin (Anonymous, 1945), D ornithine, D phenylalanine, and D glutamic acid in bacitracin A (Craig, Hausmann, & Weisiger, 1954, Lockhart & Abraham, 1954). D proline is a constituent of ergot alkaloids (Smith & Timmis, 1937). Spores of *Bacillus megatherium* contain a peptide formed from D alanine, D glutamic acid, and several other amino acids (Strange & Thorne, 1957). Piutti (1886) isolated 100 g of D asparagine from 20 kg of crude asparagine, the product of 6,500 kg of vetch seedlings. The D amide, like D amino acids, had a sweet taste.

D amino acids, though much less important than the L isomers, thus have some metabolic significance in micro organisms at least. Liver and kidney of various mammals contain an oxidase attacking many D amino acids but not their L isomers. D amino acids may thus play some part in animal metabolism also. The growth of lentil seedlings (*Erium lens*) is accelerated by L-isoleucine and inhibited by its D isomer (Nicolle, Coste Sodigné, & Diot, 1959).

## B. Amino-acids found regularly in Protein

The amino acids commonly found in protein are shown in Table 4. Most of these are monoaminomonocarboxylic acids, glycine, alanine, cysteine, valine, leucine, isoleucine, serine, threonine, methionine, phenylalanine, tyrosine, tryptophan, proline, hydroxyproline. The last two, though actually imino acids, are always considered with the amino acids. Other special features include the hydroxyl groups of serine and threonine, the methylthio group of methionine, the aromatic rings of phenylalanine and tyrosine, and the indolyl structure of tryptophan. Aspartic and glutamic acid are monoaminodicarboxylic compounds. Their amides, asparagine and glutamine, are incorporated independently

thyroxine and 3,5,3'-triiodothyronine (Roche & Jouan, 1956). Iodotyrosines may occur also in proteins of marine algae. Golenkin (1954) noted that the red alga *Bonnemaisonia asparagoides* contained organically bound iodine. Roche & Lafon (1949) found diiodotyrosine in *Laminaria flexicaulis*, which Roche & Yagi (1952) showed to incorporate radioactive iodide ( $I^{131}$ ) into mono- and diiodotyrosines. Similar results were obtained with another brown alga (*Nereocystis luetkeana*) by Tong & Chaikoff (1955) and by Scott (1954) with green, brown, and red algae (*Ulva lactuca*, *Laminaria digitata*, and *Rhodomenia palmata*). Coulson (1955b) tentatively identified thyroxine, thyronine, and 3,5-diiodothyronine by chromatography in the last named species. The red alga *Polysiphonia fastigiata* contains (Mastigh & Augier, 1949) a dihydroxybenzoic acid which may be a metabolite of dibromotyrosine.

Fowden (1959b) demonstrated synthesis of iodine-containing amino acids by higher plants. He detected 3,5-diiodotyrosine, 3,5-diiodothyronine, and 3,5,3'-triiodothyronine in salt marsh plants (*Aster tripolium* and *Salicornia perennis*) supplied with labelled iodide. Barley (*Hordeum sativum*) and the bean *Phaseolus vulgaris* also incorporated labelled iodide into 3,5-diiodotyrosine. Yeast (*Saccharomyces cerevisiae*) does not normally contain iodoamino acids, but if supplied with 3,5-diiodotyrosine incorporates it into protein (Hahermann, 1958).

Amino acids containing chlorine or fluorine are unknown as natural products. The former may well exist: chloromycetin (chloramphenicol) a *Streptomyces* antibiotic, being a derivative of a chlorinated nitrophenylserine. Plants containing fluoracetic acid, e.g. *Dichapetalum cymosum* (Marais, 1944; Badenhuizen & Slinger, 1954) and *Acacia georginae* (Oelrichs & McEwan, 1961) would be likely sources of fluorine-containing amino acids.

Phosphoserine has been isolated from proteins of animal origin (Agren, De Verdier & Glomset, 1954; Kennedy & Smith, 1954; Vladimirov, Ivanova & Pravdina, 1956) in the protein phosphatase from egg yolk; all the phosphorus seems to be associated with seryl residues (Mecham & Olcott, 1949). The amino acid sequence

—aspartic acid—phosphoserine—glycine—

occurs in chymotrypsin, choline esterase and trypsin (Schaffer, Simet, Harshman, Finkle & Drisko, 1957) and in phosphoglucomutase (Anderson & Jolles, 1957; Koblentz & Erwin, 1957). Sequences containing two to six and possibly more successive residues of phos-



Recently discovered protein amino-acids are few and of restricted distribution. New non-protein amino-acids have in contrast been found in large numbers. Some are known only from one or a few species; others apparently are generally distributed. Paper chromatography has detected numerous previously unsuspected amino-acids in plant extracts. Some of these have been isolated and identified, but many that appear distinct from known compounds still await identification. Fowden & Steward (1957a) reported 53 unidentified ninhydrin-reacting substances in species of Liliaceae. The restricted known distribution of many amino-acids, together with the comparatively few species examined, suggest that the total number of amino-acids formed by plants may be very large. Amino-acids recently recognized as natural products, or of limited known distribution, will now be considered in groups based on their chemical structure.

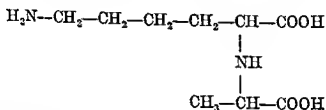
#### D. Non- $\alpha$ -Amino-acids

Several bacteria decarboxylate glutamic acid to  $\gamma$ -aminobutyric acid (Abderhalden, Fromme, & Hirsch, 1913) and aspartic acid to  $\beta$ -alanine (Ackermann, 1911); both are now recognized as constituents of higher plants. Dent, Stepka, & Steward (1947) detected  $\gamma$ -aminobutyric acid by chromatography; it was isolated later from heetroot (Westall, 1950), ryegrass (*Lolium perenne*) (Synge, 1951), and potato (Thompson, Pollard, & Steward, 1953). It is very widely distributed, occurring in flowering plants, ferns, mosses, fungi, and bacteria, often as one of the most prominent free amino-acids. Its betaine occurs in the fungus *Polyporus sulphureus* (List, 1958). Seeds of *Erysimum rupestris* (Cruciferae) contain (Kjaer & Gmelin, 1957) a glucoside yielding on hydrolysis the methyl ester of  $\gamma$ -isothiocyanatobutyric acid, a substance closely related to  $\gamma$ -aminobutyric acid. An isomer of  $\gamma$ -aminobutyric acid,  $\beta$ -aminoisobutyric acid, is formed in mammals as a breakdown product of the pyrimidine thymine (Fink, Henderson, & Fink, 1952; Fink, Cline, Henderson, & Fink, 1956). It was found in human urine by Crumpler, Dent, Harris, & Westall (1951), and isolated from vegetative storage organs of *Iris tingitana* by Asen, Thompson, Morris, & Irreverre (1959).

$\beta$ -Alanine is another widespread plant constituent, but occurs in smaller amounts than  $\gamma$ -aminobutyric acid and cannot always be detected. It occurs in leaves of lucerne (alfalfa, *Medicago sativa*) (Steward, Thompson, Mullar, Thomas, & Hendricks, 1951); in leaves and fruits of apple (*Pyrus malus*) (Hulmo & Arthington, 1950; McKee &

acid, which occurs in this plant. Some bacteria decarboxylate  $\gamma$  hydroxyglutamic acid, a known plant constituent, to  $\gamma$  amino  $\alpha$  hydroxybutyric acid (Virtanen & Hietala, 1955b). The decarboxylation product is not known in higher plants. Its isomer  $\gamma$  amino  $\beta$  hydroxybutyric acid occurs free in brains of man and other mammals (Obara, Sano, Koizumi, & Nishinuma, 1959), it is produced also by bacterial decarboxylation of  $\beta$  hydroxyglutamic acid (Umbreit & Heneage, 1953).

Crown gall tissue of *Helianthus tuberosus*, *Nicotiana tabacum*, *Parthenocissus tricuspidata*, and *Scorzonera hispanica* contains (Lloret, 1957a, b) large amounts of lysopine, an amino acid absent from normal tissues. Its structure (Biemann, Lloret, Asselineau, Lederer, & Polonsky, 1960a, b) is



Lysopine is the lysine analogue of octopine, found in octopus muscle but not recorded in plants.

Cysteic acid, formed by oxidation of cysteine, differs from aspartic acid only in the replacement of one carboxyl group by a sulphonio acid ( $-\text{SO}_3\text{H}$ ) group. On decarboxylation it yields taurine, which occurs in the red algae *Porphyra umbilicalis* and *Ptilota pectinata* (Lindberg, 1955) and *Chondrus crispus* (Young & Smith, 1958). The first two algae also contain *N* methyltaurine, di *N* methyltaurine occurs in *Gelidium cartilagineum* (Lindberg, 1955). Cysteinesulphonic acid is an intermediate (Pine, 1934; Medes, 1939) in the oxidation of cysteine in animals, its decarboxylation product hypotaurine, is an animal metabolite (Chatagner & Bergeret, 1951) but seems unknown in plants. The enzymes decarboxylating glutamic acid and cysteine acid are distinct, glutamic acid decarboxylase from radish and carrot roots having no effect on cysteine acid (Werle & Bruninghaus, 1951).

#### E. $\gamma$ -Derivatives of Glutamic acid

Done & Fowden (1952) isolated  $\gamma$  methyleneglutamic acid and  $\gamma$  methylamino glutamine from the peanut (*Arachis hypogaea*), the identifications being confirmed by comparison with synthetic material (Wales, Whiting & Fowden, 1954). Both the acid and the amide

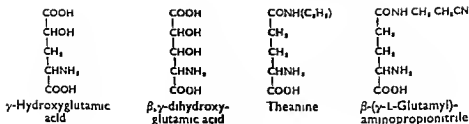


FIG. 5.

## F. Other Dicarboxylic Amino-acids

$\alpha,\epsilon$ -Diaminopimelic acid has been isolated from acid hydrolysates of *Corynebacterium diphtheriae* (Work, 1950), *Mycobacterium tuberculosis* (Asselineau & Lederer, 1950), and *Vibrio cholerae* (Blass, Le Comte, & Machebocuf, 1951). It appears to be fairly widespread among micro-organisms, including blue-green algae (Work & Dewey, 1953) and the unicellular green alga *Chlorella ellipsoidea* (Fujiwara & Akabori, 1954). It is not known from higher plants. Its  $\beta$ -hydroxy derivative occurs in the toxin (tabtoxinine) produced by *Pseudomonas tabaci* (Woolley, Schaffner, & Braun, 1952). The fern *Asplenium septentrionale* contains  $\beta$ -aminoadipic acid (Virtanen & Berg, 1954), and  $\gamma$ -hydroxy- $\alpha$ -aminopimelic acid and its lactone (Virtanen, Uksila & Matikkala, 1954). Fowden (1958c) found  $\alpha$ -aminoadipic acid in the grasses *Brachypodium sylvaticum*, *Bromus carinatus*, *Dactylis glomerata*, *Festuca heterophylla*, *Hordeum vulgare*, *Lolium perenne*, *Poa alpina*, *P. glauca*, *P. nemoralis*, and *P. pratensis*. These dicarboxylic amino-acids are shown in Fig. 6. There is evidence (Gilvarg, 1957) that in *Escherichia coli* diaminopimelic acid is synthesized via *N*-succinyldiaminopimelic acid, which probably arises (Rhuland & Soda, 1959) by the condensation of one molecule each of aspartic acid, pyruvic acid, and succinic acid.

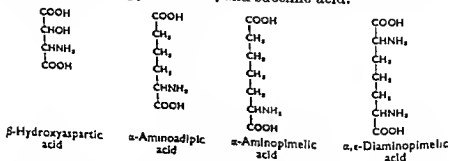


FIG. 6.

In contrast to the many derivatives of glutamic acid, few new compounds related to aspartic acid have been found in higher plants. Virtanen & Saris (1957) recorded  $\beta$ -hydroxyaspartic acid (see Fig. 6)

## AMINO-ACIDS AND BETAINES

y, 1952). Hygric acid (*N*-methylproline) occurs in alkaloids of Solanaceae (Willstätter, 1900), of *Erythroxylon coca* (Wohler, and of *Convolvulus hamadae* (Lazurevski, 1939). *N*-methyl-4-ypoline is known from *Croton gubouga* (Euphorbiaceae) on & Clewer, 1919). Nitta, Watase, & Tomiie (1958) isolated from the alga *Digenea simplex* a dicarboxylic pyrrolidine derivative they named kainic acid and characterized as 2-carboxyl-3-ymethyl-4-isopropenylpyrrolidine. Fig. 7 shows the structures of some naturally occurring pyrrolidines.

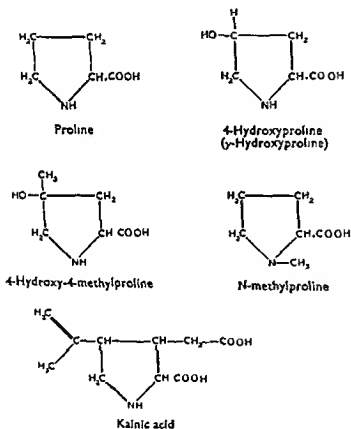


FIG. 7.

Actithiazic acid (Fig. 8), an antibiotic formed by *Actinomyces jiniiae*, is an imino acid containing a thiazole ring (Schenk & De se, 1952).

Simple piperidine carboxylic acids occur in the betel nut (seeds of : palm *Areca catechu*) These compounds, guvacine (3,4 dehydro-eridine 3-carboxylic acid) (Jahns, 1891, Freudenberg, 1918) and

## H Other Cyclic Amino-acids

Azetidine 2 carboxylic acid, a lower homologue of proline containing a ring of three carbon atoms and one nitrogen atom, was first isolated from *Coniallaria majalis* (Fowden, 1955a, 1956) and *Polygonatum officinale* (Virtanen & Linko, 1955a). It occurs in about 20 species of Liliaceae out of 89 tested by Fowden & Steward (1957a), its known distribution is restricted to Liliaceae (including *Agave* and related genera, sometimes separated as a distinct family) and Amaryllidaceae. The only other natural product reported to contain the azetidine ring is the actinomycete antibiotic nocardamine (Fig 9) (Stoll, Renz, &

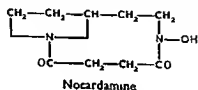


Fig 9

Brack, 1951). A related compound 4 keto azetidine 2 carboxylic acid, was stated to be formed by heating asparagine for 24 hours at 100°C in phosphate buffer of pH 6.7 (Talley, Fitzpatrick, & Porter, 1956). These authors, however, reported later (Talley *et al.*, 1959) that their compound was in fact fumaramic acid, first synthesized by Griess (1879). Various synthetic compounds have been assigned structures containing the azetidine ring. Some of these proposed structures are incorrect (King & Clark Lewis, 1951a, b, King, Clark Lewis, & Morgan, 1951) but others seem well founded (Kipping & Perkin, 1889, Staudinger, Göhring, & Schöller, 1914). The synthetic compounds are azetidine 2,4-diones, a long series has been synthesized (Ehnöther, Jucker, Rissi, Rutschmann, Schreier, Steiner, Suess, & Vogel, 1959).

No nitrogen containing ring smaller than that of azetidine 2 carboxylic acid is likely to be stable. 1 Amino cyclopropane 1 carboxylic acid, an amino acid containing a ring of three carbon atoms with the nitrogen atom in a side chain, occurs in pears (Burroughs, 1957) and in the cowberry (*Vaccinium vitis idaea*) (Vahatalo & Virtanen, 1957). Its structure, together with those of some other cyclic amino acids, is shown in Fig 10. Another amino acid with the 3 membered cyclopropane ring occurs in *Blighia sapida* (Sapindaceae), from whose seeds Hassal, Reyle, & Feng (1954) isolated two toxic compounds named hypoglycins A and B because they markedly reduced blood sugar levels. Wilkinson (1958b) identified hypoglycin A as  $\beta$  (methylene

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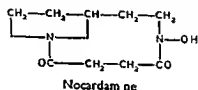


Fig 9

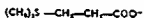
Brack, 1951). A related compound, 4-keto azetidine 2 carboxylic acid, was stated to be formed by heating asparagine for 24 hours at 100°C in phosphate buffer of pH 6.7 (Talley, Fitzpatrick, & Porter, 1956). These authors, however, reported later (Talley *et al.*, 1959) that their compound was in fact fumaramic acid, first synthesized by Gress (1879). Various synthetic compounds have been assigned structures containing the azetidine ring. Some of these proposed structures are incorrect (King & Clark Lewis, 1951a, b, King, Clark Lewis, & Morgan, 1951) but others seem well founded (Kipping & Perkin, 1889, Staudinger, Göhring, & Schöller, 1914). The synthetic compounds are azetidine 2,4-diones, a long series has been synthesized (Ebnöther, Jucker, Rissi, Rutschmann, Schreier, Steiner, Suess, & Vogel, 1959).

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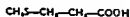
acid not known elsewhere In *Neurospora crassa* (Melville, Lich, & Ludwig, 1957) and in *Claviceps purpurea* (Heath & Wildy, 1957) ergothioneine is synthesized from histidine, not from 2 thiohistidine *Neurospora* uses sulphur from sulphate thiosulphate, cysteine, or methionine in the synthesis of ergothioneine Ergothioneine occurs in association with hereynine (the betaine of histidine) in erythrocytes of cattle seminal fluid of the hoar, the fungus *Coprinus comatus*, and the king crab *Limulus polyphemus* (Ackermann, List, & Menssen, 1959) Hereynine, recorded in *Limulus* by Ackermann & List (1958), is otherwise known only from higher fungi (Reuter, 1912, List, 1958) Ackermann and his associates suggest the following biosynthetic sequence



$\beta$  Dimethylpropiothetin, found in the red alga *Polysiphonia fastigiata* (Haas & Russel Wells, 1923) and the green alga *Enteromorpha intestinalis* (Bywood & Challenger, 1953), is the betaine of  $\beta$  methylthiol propionic acid (Fig 14)



$\beta$ -Dimethylpropiothetin



$\beta$ -Methylthiolpropionic acid

FIG 14

Oxidation products of methionine (methionine sulphone and methionine sulfoxide) and of cysteine (cysteine acid), often found in chromatograms of plant extracts, are generally regarded as artifacts arising by oxidation of the parent amino acids during analysis If they do occur naturally their unequivocal detection would be difficult

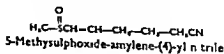
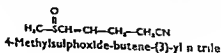
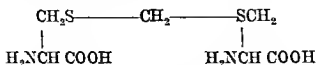


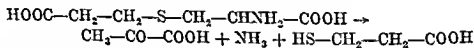
FIG 15

Other sulfoxides are known from plant tissues Schmid & Karrer (1948a b) obtained 4 methylsulphoxide butene-(3) yl nitrile and the corresponding isothiocyanate by hydrolysis of glucosides from seeds of the radish (*Raphanus sativus*) A higher homologue of the nitrile,

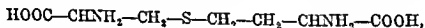
isolated directly from the seeds (Van Veen & Hyman, 1933) Its structure (Van Veen & Hyman, 1935) is



This structure has been confirmed by synthesis (Du Vigneaud & Patterson, 1936, Armstrong & Du Vigneaud, 1947) Djenkolic acid occurs also in seeds of *Psithcolobium dulce*, *P. multiflorum*, and *Albizia lophantha* (Leguminosae) (Gmelin, Hasenmaier, & Strauss, 1937) Gmelin, Strauss, & Hasenmaier (1938) isolated a new sulphur containing amino acid, *S*( $\beta$  carboxyethyl) L-cysteine, from seeds of *Albizia julibrissin* On enzymatic breakdown it formed ammonia, pyruvic acid, and  $\beta$  thiolpropionic acid

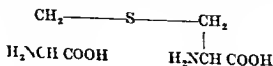


This amino acid also occurs, together with a related compound (probably *S* ( $\gamma$  carboxypropyl) L-cysteine), in seeds of *Acacia willardiana* (Gmelin, 1939) Cystathionine,



is an intermediate in the formation of methionine by *Neurospora crassa* (Horowitz, 1947, Teas, Horowitz, & Fleng, 1948) It is broken down (Gmelin, Hasenmaier, & Strauss, 1937) by an enzyme from seeds of *Albizia lophantha* to ammonia, pyruvic acid, and homocysteine ( $\text{HS-CH}_2\text{-CH}_2\text{-CHNH}_2\text{-COOH}$ ), another intermediate in methionine synthesis by *Neurospora*

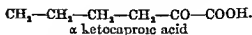
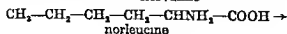
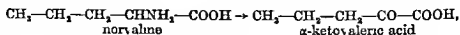
Lanthionine, a diaminodicarboxylic acid structurally resembling djenkolic acid, occurs in hydrolysates of wool but is probably an artifact not existing in the original protein (Schöberl & Wagner, 1956) It occurs in the antibiotics subtilin (Uderton & Fevold 1951) and duramycin (Shotwell, Stodola Michael, Lindenfelser Dworschach, & Prudham, 1953), the latter also contains  $\beta$  methylanthionine The structure of lanthionine is



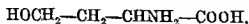
Lanthionine is not definitely known from higher plants, it is, however,



tomato roots (Boll, 1954a, b) and may thus be a normal metabolite. Norvaline and norleucine are readily metabolized in the animal body, probably by transamination to the corresponding keto-acids (Hassan & Greenberg, 1952):

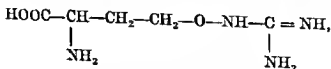


Homoserine,

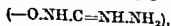


an isomer of threonine with the hydroxyl group on the  $\gamma$  carbon atom, is an intermediate in the metabolism of methionine in rats (Binkley & Du Vigneaud, 1942; Stetten, 1942), *Neurospora crassa* (Teas, Horowitz, & Fling, 1948), *Escherichia coli* (Lampen, Roepke, & Jones, 1947), and *Saccharomyces cerevisiae* (Pomper, 1953). It has been isolated as the lactone, to which it cyclizes readily, from the pea (*Pisum sativum*) (Miettinen, Kari, Moisio, Alfthan, & Virtanen, 1953). The pea also contains *O*-acetylhomoserine. Another hydroxyamino-acid,  $\gamma$ -hydroxyvaline, is known only from *Kalanchoe daigremontiana*; it appears to be absent from six other species of *Kalanchoe* (Crassulaceae) (Pollard & Steward, 1955; Pollard, Sondheimer, & Steward, 1958).

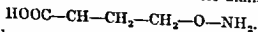
Canavanine ( $\alpha$ -amino- $\delta$ -guanidoxymybutyric acid) was discovered in seeds of *Canavalia obtusifolia* and *C. lineata* by Kitagawa & Tomiyama (1929), and recorded in soybeans (Muller & Armbrust, 1940). Its structure,



is of interest as containing the guanidinoxy group



which is rare among natural products. Damodaran & Narayanan (1940) showed that seeds of *Canavalia ensiformis* contained canavanine and an enzyme hydrolysing it to urea and another amino-acid, canaline:



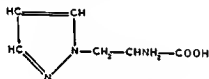
Some bacteria decompose canavanine to homoserine and guanidino (Kihara, Prescott, & Snell, 1955), another enzymatic reaction hydrolyses canavanine to *O* ureidohomoserine and ammonia (Kihara & Snell,

chromatography is reported in *Atropa belladonna* (Solanaceae) (James, 1949), *Ainus glutinosa* (Betulaceae) (Miettinen & Virtanen, 1952), *Kalanchoe blossfeldiana* (Crassulaceae) (Madan, 1956), *Phelipaea ramosa* (Orobanchaceae) (Izard, 1958), and the fern *Asplenium nidus* (Virtanen & Linko, 1955b). The presence of ornithine in watermelon (*Citrullus vulgaris*) (Kasting & Delwiche, 1957) and in flax (*Linum usitatissimum*), where it accumulates in sulphur deficiency (Coleman, 1958), is firmly established by isolation. Ornithine, rarely more than a minor constituent of the free amino acids, is prominent in the red alga *Chondrus crispus* (Young & Smith, 1958). It occurs in the antibiotic peptides gramicidin S (Synge, 1945b, Sanger, 1946) and tyrocidine (Gordon, Martin, & Synge, 1943). Ornithine, though absent from most proteins, represents 6 per cent of the nitrogen in hydrolysates of insoluble material (free of soluble constituents) from the red alga *Chondrus crispus* (Smith & Young, 1955), it was detected chromatographically and isolated from the hydrolysates, but was not found in similar preparations from other red, brown, and green algae. The protein component of kidney phosphatase is stated to contain ornithine (Lora Tamayo & Municio, 1953), it is also reported in a protein of the marine mollusc *Busycon canaliculatum* (Sbasshoua & Kwart, 1959).

$\delta$ -N acetylornithine accumulates in vegetative storage organs of some plants, forming 10 per cent of the dry weight in roots of *Corydalis ochotensis* (Manske, 1937). Reuter (1957a) recorded it as the main soluble nitrogenous compound in the storage organs (roots, tubers, and stems) of the following members of the same family (Fumariaceae): *Adlumia cirrhosa*, *A. fungosa*, *Corydalis cava*, *C. cheiranthifolia*, *C. fabacea*, *C. glauca*, *C. lutea*, *C. nobilis*, *C. ochroleuca*, *C. rosea*, *C. semper virens*, *C. solida*, *C. thalictroides*, *C. vaginans*, *Dicentra eximia*, *D. formosa*, *D. spectabilis*, *Fumaria capreolata*, *F. officinalis*. It was a minor constituent in the storage organs of some members (*Chelidonium majus*, *Glaucium flatum*, *Hylamecon japonica*, *Stylophorum diphyllum*) of the Papaveraceae, a family closely related to Fumariaceae, which some botanists consider a sub family (Fumarioideae) of Papaveraceae. All species of Fumariaceae tested had  $\delta$ -N acetylornithine as the main soluble nitrogenous constituent of the vegetative storage organs, it was absent from 17 species of Papaveraceae and from all species (over 140) of other families tested by Reuter (1957a). Virtanen & Linko (1955b) detected it in *Corydalis bulbosa* and in ferns (*Asplenium* spp.). Fowden (1955c) found large amounts of  $\delta$ -N acetylornithine in *Poa glauca*, it

Citrulline seems not to be a general constituent of protein. Klein & Tauböck (1932a) stated that it occurred in proteins from *Cucumis sativus* and other cucurbits, but did not explain how this conclusion was reached. Smith & Young (1955) reported that citrulline (detected by chromatography but not isolated) occurred regularly in hydrolysates of insoluble material from the red alga *Chondrus crispus*. No citrulline was found in similar hydrolysates from other algae (*Fucus vesiculosus*, *Ascophyllum nodosum*, *Rhodomenia palmata*, and *Ulva lactuca*). Citrulline is rarely reported from proteins of animal origin but is stated (Rogers & Simmonds, 1958) to form 6 per cent of a protein from hair follicles of the rat.

Watermelon (*Citrullus vulgaris*) contains another unusual amino-acid, isolated and identified by Noé & Fowden (1959, 1960). This compound,  $\beta$ -pyrazolylalanine (Fig. 17), is an isomer of histidine containing the first pyrazolo ring detected in a natural product. A somewhat similar alanine derivative is formed in *Phaseolus* plants treated with the herbicide 3-amino-1,2,4-triazole (Massini, 1959).



$\beta$ -Pyrazolylalanine

FIG. 17.

A lower homologue of citrulline has been isolated from seeds of *Acacia dealbata*, *Albizia julibrissin*, *Enterolobium cyclocarpum*, *Lysiloma bahamense*, *L. desmostachys*, and *Pithecolobium albicans* (Gmelin, Strauss, & Hasenmaier, 1958, 1959) and named albizziine. Its structure, 2-amino-3-ureidopropionic acid, has been confirmed by synthesis (Kjaer, Larsen, & Gmelin, 1959). Albizziine shows some structural resemblance to leucaenol (mimosine), found in *Leucaena glauca* (Mascré, 1937; Bichel & Wibaut, 1946; Hegarty, 1957) and *Mimosa pudica* (Renz, 1936; Kleipool & Wibaut, 1950). Leucaenol is  $\beta$ -(*N*-(3-hydroxy-4-pyridone))- $\alpha$ -aminopropionic acid. Like albizziine, it is known only from members of the sub-family Mimosoideae of the Leguminosae. A simpler amino-acid related to these compounds,  $\alpha,\beta$ -diaminopropionic acid, occurs in seeds of *Mimosa hemendyla* and *M. palmeri* (Gmelin, Strauss, & Hasenmaier, 1959). Its only other known natural occurrence is as a constituent of the antibiotic viomycin (Haskell, Fusari, Frohardt,

D-serine is produced by a *Streptomyces* but has no antibiotic activity (Hagemann, Pénasse, & Teillon, 1955). Structures of these compounds are shown in Fig. 18.

3,4-Dihydroxyphenylalanine, closely related to tyrosine (4-hydroxyphenylalanine), is known from *Vicia faba* (Guggenheim, 1913), species of *Stizolobium* (Miller, 1920), and *Mucuna capitata* (Yoshida, 1945); these legumes are apparently the only plants in which it is recorded. Another derivative of tyrosine, 2,4-dihydroxy-6-methylphenylalanine, is reported from *Agrostemma githago* (Caryophyllaceae) (Schneider, 1958). *O*-methyltyrosine occurs in the antibiotic puromycin formed by *Streptomyces alboniger* (Waller, Fryth, Hutchings, & Williams, 1953). *N*-methyltyrosine (surinamine) occurs in the bark of *Geoffraea surina-*

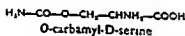
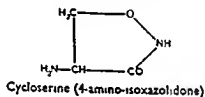
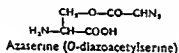


FIG. 18.

*ensis* (Leguminosae) (Winterstein, 1919) and *N*-methyltryptophan (abrine) in seeds of *Abrus precatorius* (Leguminosae) (Ghatak & Kaul, 1932; Hoshino, 1935; Cahill & Jackson, 1938). Stowe, Thimann, & Kefford (1956) also isolated *N*-methyltryptophan from these seeds but were unable, in spite of its comparatively high concentration in extracts, to detect it by chromatographic methods successful with pure solutions. This masking by other substances of a constituent which should be prominent is a warning against uncritical acceptance of chromatographic data unsupported by other techniques. The name "nhrine", used for *N*-methyltryptophan, should not be confused with nhirin, a toxic protein also found in seeds of *Abrus precatorius*. Good & Andreae (1957) found a malonyltryptophan in pea, spinach, and tomato plants.

The tyrosine commonly found in biological material is the *para* compound. *Ortho* and *meta* tyrosines are unknown in plants but there is evidence for their occurrence in animal products. Dennell (1956)

chromatographically in germinating peas by Faweett, Seeley, Taylor, Wain, & Wightman (1955), and isolated from aqueous extracts of cabbage by Jones & Taylor (1957); indolyl-3-pyruvic acid (Stowe & Thimann, 1953; Vlitos & Meudt, 1954); indolyl-3-propionic acid (Linser, Mayr, & Maschek, 1953); indolyl-3-butyric acid (Blommaert, 1954); 5-hydroxyindolyl-3-acetic acid (Udenfriend, Titus, & Weissbach, 1955). 5-Hydroxytryptophan occurs in *Chromobacterium violaceum* (Mitoma, Weissbach, & Udenfriend, 1955). Wieland & Witkop (1940) and Šorm & Keil (1951) found a hydroxytryptophan in a toxic peptide of the fungus *Amanita phalloides*. Cornforth, Cornforth, Dalglish, & Neuberger (1951) synthesized the compound from isatin and ethyl pyruvate, formulating it as  $\beta$ -3-oxindolylalanine.

Peptides of indolyl-3-acetic acid are known from natural sources. Tissues of several plants synthesize indolyl-3-acetylaspargic acid when supplied with IAA (Good, Andreae, & Van Ysselstein, 1956); indolyl-3-acetylglutamine occurs in very small amounts in normal human urine,

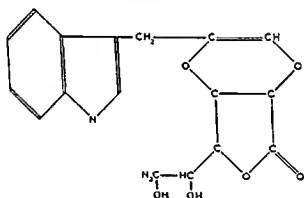


FIG. 19.

its output being greatly increased in pathological states involving a large excretion of IAA (Jepson, 1956). Procházka, Šanda, & Šorm (1957) isolated from cabbage a compound yielding ascorbic acid and IAA on hydrolysis. They proposed the structure shown in Fig. 19 for this substance, which they called ascorbigen, a name used earlier by other writers for ill-defined complexes of protein with ascorbic acid. *Bacillus megatherium* incorporated added indolyl-3-propionic acid into peptides with alanine, serine, and threonine (Tabone, 1958).

Amides of non-nitrogenous acids are known from various plants. Little is known of their metabolism but some have attracted attention

## K Betaines

The name 'betaine', coined by Scheibler (1869a) for a substance which he isolated from the juice of sugar beet (*Beta vulgaris*), is now applied more generally to a family of *N* methyl internal anhydrides of amino or imino acids. They can also be regarded as quaternary ammonium bases carrying a carboxyl group, this zwitterion structure expresses their chemical properties better than the anhydride structure

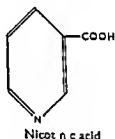
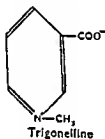
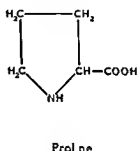
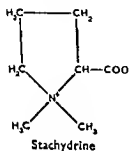
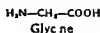
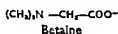


FIG 20

The betaines of many common amino acids are unknown as natural products, and only a few occur widely. The best known are trigonelline, stachydrine, and glycine betaine derived respectively from nicotinic acid, proline, and glycine (Fig 20).

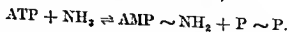
Glycine betaine is widely distributed among flowering plants, occurring in all organs; it may form 5 per cent of the dry weight in leaves (Klein, Krich Pollauf & Soos 1931; Cromwell & Rennie 1953). It occurs also in the fungi *Boletus edulis* (Reuter 1912) and *Amanita muscaria* (Kunze 1914) and in bacterial cultures (Cromwell & Rennie 1954a). Stachydrine first isolated from tubers of *Stachys tubifera* (Von Planta & Schulze 1893) is known from many flowering plants, as is trigonelline discovered by Jahns (1885) in *Trigonella foenum-graecum*. Other betaines are known only from a few species: Betonicine and

ornithine, and tryptophan. Barrenscheen & von Vályi-Nagy (1942) reported that in a homogenate of etiolated wheat seedlings methionine supplied methyl groups for the conversion of glycine to its betaine. Cromwell & Rennie (1954a) did not confirm this observation, but found that choline infiltrated into leaves of *Atriplex patula* or *Beta vulgaris* was oxidized to betaine; homogenates were inactive, enzymatic activity apparently requiring intact cellular structures. Leete, Marion, & Spenser (1955a) supplied seedlings of *Medicago sativa* with  $C^{14}$ -labelled ornithine and found no evidence of its conversion to stachydrine. Wiehler & Marion (1958) showed, however, that these seedlings transformed ornithine to stachydrine if supplied with pyridoxal and folic acid. Seedlings given ornithine alone formed glutamic acid; addition of pyridoxal permitted its conversion to proline, which with added folic acid was methylated to stachydrine. The seedlings apparently lacked adequate supplies of co-factors catalysing its synthesis in the mature plant. This work establishes ornithine as a precursor of stachydrine *in vivo*; more generally it stresses that negative results in biosynthetic studies have no significance unless the test plants can actively synthesize the relevant compounds. Use of inactive plants may explain some unresolved contradictions in this field.

Little is known about the metabolic breakdown of betaines. The betaine content of germinating seed-halls of *Beta vulgaris* falls from 7 mg/g to 2 mg/g in four days; the decrease is not due to mould action or to loss of betaine by diffusion in water, but represents a metabolic conversion (Simenauer, 1957).

enzymes prepared from them (Kretovich, Bundel, & Gunar, 1955). A similar synthesis of aspartic acid from oxalacetic acid in homogenates of pea seedlings was reported by Kretovich, Bundel, & Aseyeva (1951). Bulen (1956) prepared from leaves of corn (*Zea mays*) a glutamic acid dehydrogenase dependent on diphosphopyridine nucleotides, but apparently not on any metal. Glutamic acid is the only amino-acid for which dehydrogenases are known in higher plants but *Bacillus subtilis* contains a very specific DPN-dependent dehydrogenase synthesizing alanine from ammonia and pyruvic acid (Wiame & Piérard, 1955; Fairburst, King, & Sewell, 1956). Aspartic acid and alanine could arise by the amination of oxalacetic acid and pyruvic acid respectively; there is some evidence that they are synthesized in this way in plants. Kretovich & Bundel (1950) demonstrated a considerable synthesis of alanine on addition of ammonium pyruvate to extracts of etiolated pumpkin seedlings, but it may have been formed by transamination rather than by direct amination of pyruvic acid. Jacobi (1957) found that in the green alga *Ulva lactuca* both aspartic and glutamic acids were formed by direct amination of the corresponding keto-acids. The direct amination of pyruvic acid by ammonia to form alanine is catalysed by a highly purified enzyme from mitochondria of rat liver (Berezovskaya, 1958; Kaplanski & Berezovskaya, 1958). These authors demonstrated considerable synthesis of alanine in systems without transaminase activity. Fraustadt (1959) observed that anaerobiosis greatly increased direct synthesis of alanine in *Mucor racemosus*, probably by removal of respiration as a competitor for pyruvate.

In some micro-organisms amination of keto-acids to amino-acids is carried out by adenylyl amidate, formed in the following reaction (Katunuma, 1958; Ellfolk & Katunuma, 1959):



This enzymatic reaction was demonstrated in *Mycobacterium avium*, *Leuconostoc mesenteroides*, and *Escherichia coli*. It occurred very actively in rhizobia from leguminous root-nodules, but was absent from soybean roots and from all animal tissues tested. Enzymatic formation of aspartic acid from fumaric acid by liver preparations was reported by Jacobsen, Tapadinhas, & Pereira (1935). An aspartase from *Escherichia coli* is stated (Jacobsen & Soares, 1936) to catalyse the addition of ammonia, hydroxylamine, and hydrazine to fumaric acid, forming aspartic acid, a hydroxyaspartic acid, and diamino-succinic acid.

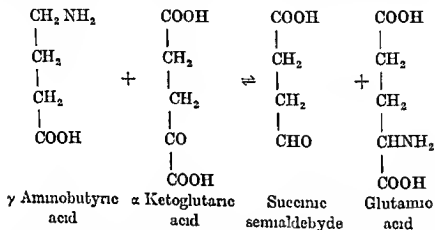


cysteic acid ( $\text{SO}_3\text{H CH}_2\text{CHNH}_2\text{COOH}$ ) (Bychkov, 1939, Cohen, 1940)

In animal tissues the aspartic acid alanine transamination requires two distinct enzymes, being in fact the sum of the first two reactions given above (Green, Leloir, & Nocto, 1945, O'Kane & Gunsalus, 1947) Wilson, King, & Burris (1954) demonstrated alanine oxalacetic acid transamination in preparations from barley and lupin seedlings, transamination between methionine and pyruvic acid was also demonstrated with preparations from mung bean seedlings. It is not clear whether these transformations occurred directly or represented the summation of more than one independent reaction. Cruickshank & Isherwood (1958) found that transaminations from glutamic acid to pyruvic acid and to oxalacetic acid are catalysed by distinct enzymes in wheat germ. Enzymes (known either as "aminopherases", the term preferred by the discoverers, or "transaminases", the term mostly used by writers in English) which catalyse the transamination reactions occur in many groups of organisms. They were reported in various plants by Virtanen & Laine (1938, 1941), Adler, Gunther, & Everett (1938), Damodaran & Nair (1938), Kritzmann (1939), Albaum & Cohen (1943), Rautanen (1946), and Leonard & Burris (1947).

Most of the naturally occurring amino acids that have been tested take part in transamination. Albaum & Cohen (1943) showed that enzymes from oat seedlings catalysed transamination to  $\alpha$  ketoglutaric acid from alanine, aspartic acid, and cysteic acid. Stumpf (1951), working with dialysed aqueous extracts from seedlings of bean, lupin, pea, and pumpkin, demonstrated transamination to  $\alpha$  ketoglutaric acid from numerous amino acids, including alanine,  $\gamma$  aminobutyric acid, aspartic acid, isoleucine, leucine, norvaline, and valine. Wilson, King, & Burris (1954) extended still further the range of transaminations catalysed by enzymes from plant tissues. Their work was particularly interesting for the techniques used, chromatographic methods being supplemented by studies of reactions between substrates labelled with  $\text{N}^{15}$  and with  $\text{C}^{14}$ . They showed glutamic acid to be formed by the transfer to  $\alpha$  ketoglutaric acid of an amino group from the following amino-acids: alanine, arginine, aspartic acid, asparagine, arginine, cysteic acid, cysteine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, serine, tryptophan, tyrosine, valine,  $\alpha$ -aminobutyric acid,  $\gamma$  aminobutyric acid and ornithine. The most active transaminations were between  $\alpha$  ketoglutaric acid and alanine, arginine, aspartic acid, and cysteic acid as donors of amino groups. Most of the metabolically important amino acids thus form glutamic acid by

found by Barnes & Naylor (1959) to be almost as good as nitrate (the best nitrogen source tested) for isolated roots of *Pinus serotina*. Citrulline was also effective as the sole source of nitrogen. Arginine, ornithine, urea, and aspartic acid supported fair growth, the nitrogen of glutamic acid was apparently unavailable, suggesting that in the roots it was not decarboxylated to  $\gamma$  aminobutyric acid. Scott & Jakoby (1958) showed transamination between  $\gamma$  aminobutyric acid and  $\alpha$  ketoglutaric acid to conform to the equation



In extracts of barley and wheat seedlings Kretovich & Galas (1959) found a rapid transamination of amino groups from  $\gamma$  aminobutyric acid to oxalacetic acid and pyruvic acid, forming aspartic acid and alanine.

Formation of amino acids by transamination implies the presence of the appropriate keto acids, or possibly of aldehydes replacing them as acceptors of amino groups. Oxalacetic acid and  $\alpha$  ketoglutaric acids are likely, as intermediates in the tricarboxylic acid cycle, to be available in actively metabolizing tissues. This applies also to pyruvic acid, the end product of glycolysis. Glyoxylic acid ( $\text{CHO}-\text{COOH}$ ) has been found in various plants since Brunner & Chuard (1886) recorded it in young fruits of grape (*Vitis vinifera*) and gooseberry (*Ribes grossularia*). It is formed in preparations from higher plants by the enzymic breakdown of glycine (Robinson & Brown 1952) and of allantoin acid (Fischer & Brunel 1937b; Kolesnikov 1950). Keto analogues of aspartic acid, glutamic acid, alanine and glycine are thus widespread in plants. Kolesnikov (1954) found that extracts of barley seedlings animated glyoxylic acid to glycine; the reaction was stimulated by glutamic acid which probably furnished the necessary amino groups by transamination. Serine is formed enzymatically from glycine and

formaldehyde in preparations from seedlings of corn (*Zea mays*), both pyridoxal phosphate and tetrahydrofolic acid are required as co enzymes (Hauschild, 1959)

The plants containing the  $\gamma$  substituted glutamic acids are known, in some cases at least, to produce their keto analogues also  $\gamma$  Methylene  $\alpha$  ketoglutaric acid has been isolated from leaves of tulip (*Tulipa gesneriana*) (Towers & Steward, 1954) and from seedlings of peanut

TABLE 6

*Keto-acids known or suspected to be intermediary metabolites, but not necessarily occurring in detectable amounts in tissues*

<i>Keto-acid</i>	<i>Corresponding amino acid</i>	<i>Organism</i>
$\alpha$ Ketobutyric	$\alpha$ Aminobutyric	<i>Escherichia coli</i> (1)
Acetoacetic	$\beta$ Aminobutyric	Flax ( <i>Linum usitatissimum</i> ) (2)
Succinic semialdehyde	$\gamma$ Aminobutyric	<i>Pisum sativum</i> (3), <i>Endomycopsis carialis</i> (4), <i>Hordeum sativum</i> (5)
Aspartic $\beta$ semialdehyde	$\alpha \gamma$ Diaminobutyric	Yeast (6)
Glutamic $\gamma$ semialdehyde	Ornithine	<i>Neurospora crassa</i> (7)
$\alpha$ Ketoisovaleric	Valine	<i>Escherichia coli</i> (8)
$\alpha$ Keto $\beta$ methylvaleric	Isoleucine	<i>Neurospora crassa</i> (9), <i>Escherichia coli</i> (10)
$\alpha$ Keto $\epsilon$ aminocaproic	Lysine	Rat (11)
Imidazolepyruvic	Histidine	Mussel ( <i>Mytilus edulis</i> ) (12)
$\alpha$ Keto- $\gamma$ methylthiol butyric	Methionine	Mung bean ( <i>Phaseolus</i> sp.) (13)
Phenylpyruvic	Phenylalanine	<i>Escherichia coli</i> (14), <i>Salvia splendens</i> (15)
<i>p</i> Hydroxyphenyl pyruvic	Tyrosine	<i>Escherichia coli</i> (14) <i>Salvia splendens</i> (15)

*References* 1 Fromageot & Desnuelle 1942 2 Johnston, Racusen, & Bonner 1954, 3 Miettinen & Virtanen, 1953b 4 Kating, 1954 5 Kretovich & Galas 1959 6 Black & Wright 1955b 7 Vogel & Bonner 1954 8 Umbarger & Magasanik, 1951 9 Wagner & Bergquist 1955 10 Abelson 1954a 11 Rothstein & Miller 1954 12 Roche Thorpe & Glahn 1954 13 Wilson Burris & King, 1954, 14 Simonson, Tatum, & Fruton 1947 15 McCalla & Neish, 1959b.

Pyridoxamine is an effective amino-group donor in a plant transaminase system (Wilson, King, & Burris, 1954). These workers also demonstrated a requirement for pyridoxal phosphate in the glutamic acid-glycine transamination of tobacco leaves. It is usually assumed that all plant transaminases require pyridoxal phosphate as co-enzyme, but this conclusion is based mainly on analogy with data for animal or bacterial

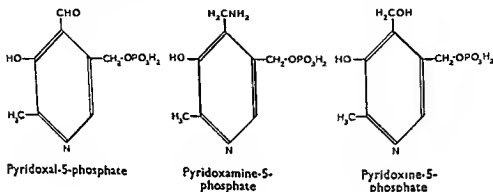


FIG. 21.

enzymes. Enzymes from *Escherichia coli* catalyse a reversible transamination between pyridoxamine and  $\alpha$ -ketoglutaric acid (Gunsalus & Tonzetich, 1952). Pyridoxine phosphate appears to combine with the active groups of the enzyme without reacting further, thus inhibiting transamination. Deoxypyridoxine phosphate has a similar effect (Meister, Sober, & Peterson, 1954). Kretovich & Yakovleva (1957) found that in a homogenate from pea seedlings formation of glutamic acid by transamination from aspartic acid was stimulated by the addition of magnesium phosphate and adenosine triphosphate. The nature of the ATP effect was not entirely clear.

#### D. The central position of Glutamic Acid in Amino-acid Metabolism

Many studies on intact plants and on isolated tissues or enzyme systems have shown that the dicarboxylic amino-acids, particularly glutamic acid, occupy a key position in the metabolic transformations of nitrogenous substances. Reasons for this are apparent in the scheme below, which summarizes the relation between the dicarboxylic amino-acids, and the tricarboxylic acid cycle, a major energy-yielding metabolic pathway in the catabolism of carbohydrate and fat.

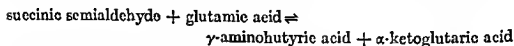
in the nitrogenous metabolism of tomato plants (MacVicar & Burris, 1948), ripening ears of wheat and seedlings of lupin, maize, and pea (Kretovich & Bundel, 1949), the unicellular green alga *Scenedesmus obliquus* (Alg  us, 1951), carrot roots (Menoret, 1957), and leaves of wheat (Carles, 1958)

Warburg & Krippahl (1958) found glutamic acid to be closely related to photosynthesis in *Chlorella*. The primary reaction of photosynthesis could not, however, be a carboxylation of  $\gamma$  aminobutyric acid, as its accumulation inhibited photosynthesis. Sivaramakrishnan & Sarma (1954, 1956) found glutamic acid to be a very active metabolite in germinating seeds of green gram (*Phaseolus* sp.). Glutamic acid uniformly labelled with  $C^{14}$  was supplied to seedlings germinating in sterile culture. After 72 hours 95 per cent of the added amino acid was catabolized, most of its carbon appearing as carbon dioxide, some carbon appeared in aspartic acid and asparagine, and a little in arginine and proline. Conversion of glutamic acid to aspartic acid involved thiamin, which probably took part as cocarboxylase in the decarboxylation of  $\alpha$  ketoglutaric acid to succinic aldehyde. Dunn, Camien, Shankman, & Block (1948) compared the total amounts of ten amino acids (free and combined in protein) in seeds and seedlings of soybean (*Glycine max*) and lupin (*Lupinus angustifolius*). In seedlings receiving no external supply of nitrogen, much of the glutamic acid of the seed proteins was converted to aspartic acid. The data of Schulze & Castoro (1903) and of Balicka Iwanowska (1903) indicate net synthesis of aspartic acid during germination of *Lupinus luteus*.

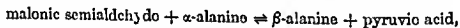
Glutamic acid is synthesized from labelled glucose by germinating seedlings. Champigny (1958a) supplied glutamic acid, labelled with  $C^{14}$  in position 1 or in positions 3 and 4, to developing plants of *Bryophyllum daigremontianum* (Crassulaceae), which were analysed 6 hours later. Part of the glutamic acid remained unchanged, part was incorporated into protein, part was transformed to glutamine or to  $\gamma$  aminobutyric acid, and part appeared in proline via pyrrolidonecarboxylic acid. Apart from these expected products  $C^{14}$  from the glutamic acid was found in a wide range of acids related to the tricarboxylic acid cycle, and in several amino acids (aspartic acid, alanine, glycine, histidine, tyrosine, valine). The carbon skeleton of glutamic acid is thus broken down, probably via decarboxylation to  $\alpha$  ketoglutaric acid, and its carbon atoms distributed into many different compounds.

Fowden & Bryant (1959) supplied  $C^{14}$  labelled aspartic acid to detached leaves of *Contallaria majalis* (lily of the valley, Liliaceae). In

Schumaeber, 1950; Beevers, 1951; Werle & Bruninghaus, 1951; Miettinen & Virtanen, 1953a; Suzuki & Takakuwa, 1957) demonstrated the enzymatic decarboxylation of glutamic acid by preparations from higher plants. Kulkarni & Sohoni (1956) found dry seeds of *Dolichos lablab* to be a very rich source of glutamic acid decarboxylase; high concentrations of the enzyme were also found in seeds of two other legumes, *Vigna catjang* and *Phaseolus aureus*. Rohrich & Rasmus (1956) showed the enzyme to be present in wheat germ and rye germ; as in other species pyridoxal phosphate acted as co-enzyme. *Chlorella* has a very active glutamic acid decarboxylase (Warburg, Klotz, & Krippahl, 1957). In some cases the product of decarboxylation was identified as  $\gamma$ -aminobutyric acid (Hase & Schumacher, 1950; Beevers, 1951; Kulkarni & Sohoni, 1956).  $\gamma$ -Aminobutyric acid also arises *in vivo* by transamination from glutamic acid:



This reaction is known in brain (where glutamic acid and the related compounds glutamine and  $\gamma$ -aminobutyric acid are very active metabolites), liver, and micro-organisms (Bessman, Rossen, & Layne, 1953; Roberts & Bregoff, 1953; Scott & Jakoby, 1958). A similar reaction,

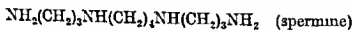


occurs in *Pseudomonas* (Nishizuka, Takeshita, Kuno, & Hayaishi, 1959).

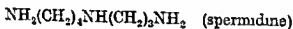
It was generally assumed, when  $\gamma$ -aminobutyric acid was first recognized as a widespread plant constituent, that it arose only in the pathways leading from glutamic acid to simpler substances. Its rôle in rat brain (Kometiani & Klein, 1953, 1955, 1956) and in tissue cultures derived from secondary phloem of the carrot root (Steward, Bidwell, & Yemm, 1956) seems more active than would be expected on this assumption. Kometiani & Klein (1953, 1955, 1956) found that a homogenate of rat brain formed ammonia when incubated with ions of potassium, magnesium, and phosphate, together with glutamic acid,  $\gamma$ -aminobutyric acid or  $\beta$ -alanine, and inosine monophosphate or inosine triphosphate. The decomposition of the amino-acids was greatly accelerated by inosine monophosphate. The authors suggested that the amino groups of the amino-acids are used in resynthesis of the adenylic system. The formation of ammonia was attributed to a deaminase acting on adenylic acid. The synthesis of adenylic acid was checked by spectrophotometry and by electrophoresis on paper.

amino acids, which are probably formed by the interaction of ammonia or other reduction products of nitrate with metabolites derived from the primary products in the fixation of carbon dioxide

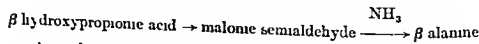
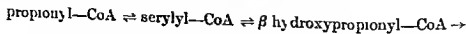
$\beta$  Alanine arises by the bacterial decarboxylation of aspartic acid (Aekermann, 1911, Virtanen, Rintala, & Laine, 1938) It has been assumed, without conclusive evidence, that higher plants form it in the same way Decarboxylation of aspartic acid to an unidentified product is reported for squash (*Cucurbita*) fruit (Rogers, 1955) and for pea shoots (Viettinen, 1957) Naylor & Tolbert (1958) studied the metabolism of  $C^{14}$  labelled aspartic acid in leaves, stems, and roots of 16 higher plants without detecting any formation of  $\beta$  alanine Another route to  $\beta$  alanine is known in bacteria Razin, Bachrach, & Gery (1958) showed that *Pseudomonas aeruginosa* rapidly oxidized the long chain amines



and



with the production of  $\beta$  alanine It was formed also from 1,3 diamino-propane but not from putrescine The metabolic relations of spermine remained obscure until recently, although it was isolated as the crystalline phosphate from human semen by Vauquehn (1791) Its synthesis in *Escherichia coli* involves S adenosylmethionine and putrescine (Tabor, Rosenthal, & Tabor, 1958) Spermine occurs in many animal tissues, and in yeast (Dudley & Rosenheim, 1925)  $\beta$  Alanine figures in animal metabolism as a late product in the breakdown of the pyrimidine uracil, its immediate precursor is  $\beta$  ureidopropionic acid (Fink, Fink, & Henderson, 1952, Batt & Exton, 1956, Canellakis, 1956) It is also the end product of a suggested pathway (Rendina & Coon, 1957) for the breakdown in animal tissues of propionic acid, itself a metabolite of valine The sequence suggested is

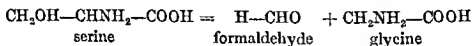


$\gamma$  Aminobutyric acid arises in several metabolic sequences It is formed by a strain of *Pseudomonas fluorescens* from pyrrolidine and from putrescine, either compound serving as sole source of nitrogen for the organism (Jakoby & Fredericks, 1959) Pyrrolidine (Pictet & Court, 1907) and putrescine (Cromwell, 1943b, Coleman & Richards, 1956) are both constituents of higher plants In some animal tissues

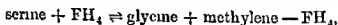
glutamic acid gives rise to proline and (presumably via ornithine) to arginine. In *Escherichia coli* *N* acetyl derivatives of glutamic acid are involved, the probable sequence being as shown in Fig. 23. *N*-acetylglutamic acid is formed in *Escherichia coli* (Maas, Novelli, & Lipmann, 1953) and in *Clostridium luyteri* (Stadtman, Katz, & Barker, 1952).

#### F. Formation of Glycine, Alanine, and Serine

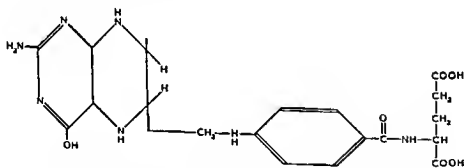
In animal tissues glycine and serine are readily converted to one another (Leuthardt & Glasson, 1942; Shemin, 1946). The first-named workers formulated the interconversion of glycine and serine as:



It is now realized that the formaldehyde in this equation can be replaced by various members of the pool of active  $\text{C}_1$  compounds. The enzymatic reaction is now written (Blakely, 1958):



where  $\text{FH}_4$  represents tetrahydrofolic acid (Fig. 24).



5, 6, 7, 8-Tetrahydrofolic acid (Huennekens, Osborn, & Whiteley, 1958)

FIG. 24.

McConnell & Bihnski (1959) injected formate and glycine labelled with  $\text{C}^{14}$  into the stems of wheat plants, and found significant radioactivity in the serine of proteins in the developing grain. Their results suggest formation of serine by condensation of glycine with formate or a  $\text{C}_1$  compound derived from it. Glycine and serine also arise from separate precursors, probably the corresponding keto-acids, glyoxylic acid and hydroxypyruvic acid. Glyoxylic acid, as already mentioned, is probably widespread in plants. Hydroxypyruvic acid is less well-known as a plant constituent, but is recorded (Virtanen & Alfthan, 1954) from the fern *Asplenium septentrionale*. It is formed by the oxidation of glyceric

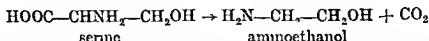




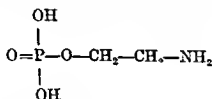
the synthesis from indole and serine of tryptophan and of indolyl acetic acid (presumably formed from tryptophan)

An enzyme from yeast catalyses the synthesis of *S* methylcysteine from serine and methyl mercaptan (Wolff, Black, & Downey, 1956) Schlossman & Lynen (1957) reported a similar synthesis of cysteine from serine and hydrogen sulphide in yeast

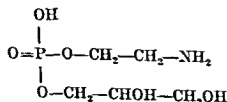
Serine is decarboxylated to aminoethanol in the rat (Stetten, 1942) and in bacteria (Nord, 1919)



There is evidence for the same reaction in tomato roots where the enzymatic decarboxylation probably requires pyridoxal phosphate (Boll, 1954b) Aminoethanol was first recognized (Trier, 1911, 1913) as a constituent of seed phosphatides The free base is reported in etiolated wheat seedlings (Steensbølt, 1946) It occurs in the antibiotics xanthomyein A (Rao, Peterson, & Van Tamelen, 1955) and gramicidin (Synge 1945a) and in the esters phosphorylaminoethanol



and glycerylphosphorylaminoethanol



Methylaminoethanol and dimethylaminoethanol occur as esters of complex non nitrogenous acids in the alkaloids of the bark of *Erythrophloeum guineense* (Leguminosae) (Faltis & Holzinger, 1939, Blount Openshaw & Todd 1940) These alkaloids have attracted attention since they were first studied scientifically (Gallois & Hardy, 1875 1876) as they are local anaesthetics and at the same time affect the heart in the same way as the cardiac glycosides They differ greatly in structure however from the steroids with unsaturated lactone rings which characterize the cardiac glycosides The methylated aminoethanols are of more general interest as precursors of choline This

*patula* and *Beta vulgaris* (Cromwell & Rennie, 1953), and probably in tobacco (Byerrum, Sato, & Ball, 1956)

Choline may also be acetylated to acetylcholine, an ester with marked physiological effects in animals. Substances pharmacologically resembling acetylcholine are reported in various fungi and higher plants. The identification is not always certain, but acetylcholine seems to occur in some species, e.g. the fungus *Lactarius blennius* (Oury & Bacq, 1937) and the nettle *Urtica urens* (Emmelin & Feldberg, 1947)

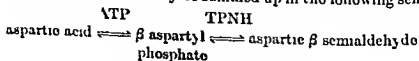
### I. Methylation by Glycine and Methionine

These amino acids are effective donors of methyl groups in alkaloid synthesis (see Chapter 12). Methionine also supplies methyl groups in the synthesis of lignin in barley and tobacco plants (Byerrum, Flokstra, Dewey, & Ball, 1954). The reaction is a transmethylation, methionine methyl groups doubly labelled with  $C^{14}$  and deuterium being incorporated into lignin with little change in the D/ $C^{14}$  ratio. In oat seedlings methionine is oxidized to methionine sulphoxide, both the amino acid and its sulphoxide transfer methyl groups to protopectin and pectin (Sato, Byerrum, Albersheim, & Bonner, 1958). The sulphoxide transfers methyl groups to methionine, forming *S*-methylmethionine, which also transfers methyl groups to pectin and protopectin, but is less active than methionine and methionine sulphoxide. Methionine provides a methyl group in the synthesis of ergosterol by yeast (Alexander, Gold, & Schwenk, 1957). The methyl group is transferred after formation of *S*-adenosylmethionine (Parks, 1958). The requirement for ATP in transmethylation suggests the general occurrence of similar intermediates (Borsook & Dubnoff, 1947a).

### J. Aspartic Acid, Homoserine, and Threonine

These amino acids are metabolically related in micro organisms, studies on yeast by Black and his co-workers in U.S.A. and on *Escherichia coli* by Cohen and his co-workers in France, have clarified the main outline of the interconversion (Black & Gray, 1953, Black & Wright, 1955a, b, c, Cohen & Hirsch, 1953, Hirsch & Cohen, 1953, Cohen, Hirsch, Wiesendanger & Nisman, 1954, Nisman, Cohen, Wiesendanger, & Hirsch, 1954).

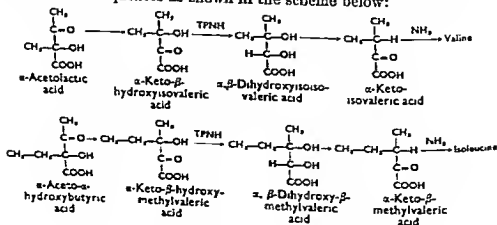
The results of this work may be summed up in the following scheme

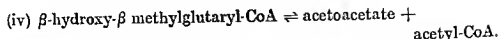
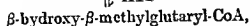
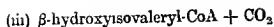


cyclizes to form dihydro-*orotic acid*, a close precursor of *orotic acid* and other pyrimidines (Wu & Wilson, 1956). The reactions involved are summarized in Fig. 27. In *Neurospora* other amino-acids seem to be precursors of pyrimidines, as pyrimidine-requiring mutants use threonine or  $\alpha$ -aminobutyric acid but not aspartic acid (Fairley, 1954).

### K. Valine, Leucine, Isoleucine

Valine and leucine appear to be metabolically more closely related to one another than to isoleucine. There is evidence that  $\alpha$ -ketovaleric acid (the keto analogue of valine) is aminated to form valine, and can also condense with an acetate unit to form an intermediate which, on decarboxylation, gives the keto analogue of leucine (Abelson, 1954a). These sequences are consistent with the observation (Arreguin, Bonner, & Wood, 1951) that the carbon of labelled acetate supplied to the guayule plant (*Parthenium argentatum*) appeared largely in valine and leucine. Normal strains of *Escherichia coli* form isoleucine and valine by transamination to the corresponding keto-acids, which accumulate in mutant strains lacking the transaminase (Rudman & Meister, 1953; Adelberg & Umharger, 1953). In mutant strains of *Escherichia coli* and *Neurospora crassa* unable to form valine and isoleucine there accumulate respectively  $\alpha, \beta$ -dihydroxyisovaleric acid and  $\alpha, \beta$ -dihydroxy- $\beta$ -methylvaleric acid. These dihydroxy acids are analogous to the keto-acids that accept amino-groups by transamination to form valine and isoleucine, and precede them in the synthetic sequence in normal strains of the micro-organisms (Myers & Adelberg, 1954; Adelberg, Coughlin, & Barratt, 1955). Several steps in the biosynthesis of valine and isoleucine have been demonstrated with cell-free extracts of *Neurospora crassa* by Wagner, Radhakrishnan, & Snell (1958), who formulate the sequences as shown in the scheme below:

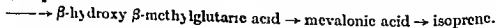
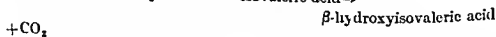
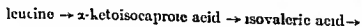




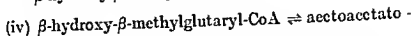
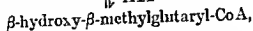
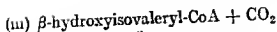
$\beta$ -Hydroxy- $\beta$ -methylglutaric acid is the esterifying acid (dicrotalic acid) in a pyrrolizidine alkaloid from *Crotalaria dura* (Leguminosae) (Adams & Van Duuren, 1953), and occurs in seeds of flax (*Linum usitatissimum*, Linaceae) (Klosterman & Smith, 1954). Millerd & Bonner (1954) showed it to be formed in small amounts in plant systems from acetoacetic acid and acetyl-CoA. Johnston, Racusen, & Bonner (1954), using enzyme systems from stem apices of flax, demonstrated the formation of  $\beta$ -hydroxy- $\beta$ -methylglutaric acid. In each case the acids were probably formed as the CoA derivatives; both reactions required adenosine triphosphate as a source of high energy phosphate. Kuzin & Nevrayova (1941) described a somewhat similar condensation of acetone and acetaldehyde to  $\beta$ -hydroxyisovaleraldehyde. This synthesis was, however, performed *in vitro* and may have no direct relation to the biosynthetic sequence.

The  $\text{C}_5$  hydroxy-acids leading to the formation of isoprene precursors can thus arise either in catabolism of branched-chain amino-acids, or by condensation of  $\text{C}_3$  and  $\text{C}_2$  units which may come from carbohydrate breakdown or, in the plant, from photosynthesis. The relative importance of these different routes to isoprene may vary in different organisms.

The intermediates of interest in this sequence are  $\beta$ -methylcrotonyl-CoA and  $\beta$ -hydroxymethylglutaryl-CoA, which are possible precursors of rubber in guayule (*Parthenium argentatum*) (Johnston, Racusen, & Bonner, 1954). An essentially similar pathway from leucine to carotenoids has been demonstrated in the mould *Phycomyces blakesleeana* by Chichester, Yokoyama, Nakayama, Lukton, & MacKinney (1959). The formation of isoprene proceeds by the following steps:



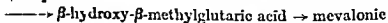
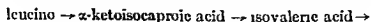
Radioactive carbon supplied as leucine was detected in carotene; the labelling was somewhat diluted, probably by carbon from the aceto-



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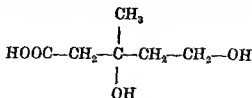
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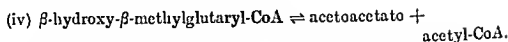
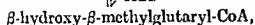
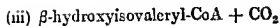
Mevalonic acid is  $\beta,\delta$ -dihydroxy- $\gamma$ -methylvaleric acid:



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The branched-chain amino-acids are thus involved in the synthesis of important non-nitrogenous compounds. Many substances physiologically active in animals, including vitamin D, sex hormones, cardiac poisons, and carcinogens, are sterols. Their functions in plants are less well known. Carotenoids are widespread in plants; they are invariably associated with chlorophyll and occur also in many fungi lacking this pigment and incapable of photosynthesis. Their functions are again better understood in animals, where carotenoids include vitamin A and the retinenes (substances concerned with the physiology of vision), than in plants. The phytol side-chain of chlorophyll is a terpene derivative, but its function in photosynthesis, like that of the carotenes and xanthophylls associated with chlorophyll, remains obscure.

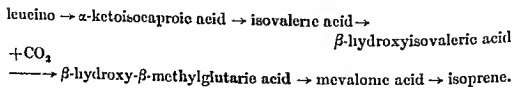
The terpenes found in essential oils and resins resemble the alkaloids in their sporadic occurrence in different groups of plants, in the complexity of their structure, and in their lack of obvious function; they



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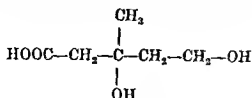


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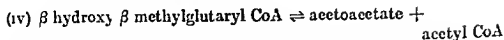
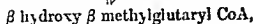
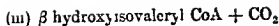
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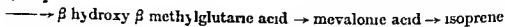
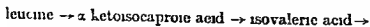
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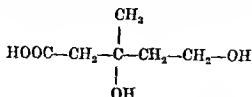
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The terpenes found in essential oils and resins resemble the alkaloids in their sporadic occurrence in different groups of plants, in the complexity of their structure, and in their lack of obvious function; they

differ in containing no nitrogen. It is sometimes stated that alkaloidal plants rarely produce essential oils, a generalization supported by the rarity of alkaloids in some of the main families producing essential oils (e.g. Pinaceae, Myrtaceae, Labiatae). Others, however, contain a few alkaloidal species (e.g. Compositae, Umbelliferae) and some (e.g. Lauraceae, Rutaceae) are prominent sources of both essential oils and alkaloids. The leaves of the three species of *Duboisia*, all notable alkaloid-producing plants, contain rather large amounts of the triterpenoid ursolic acid (Trautner & Neufeld, 1947). Some alkaloids, e.g. these of *Aconitum* and *Delphinium* (Ranunculaceae) and *Nuphar* (Nymphaeaceae), are indeed closely related chemically to the terpenes. The steroidal alkaloids of *Solanum*, *Veratrum*, and *Calotropis* may also be biosynthetically related to isoprene. Essential oils are closely related to carotenoids and many alkaloids to amino-acids. Materials for the synthesis of both groups of byproducts are therefore likely to be available in all plants. Any rigid relation between their production and plant classification is unlikely, though correspondences are often apparent between the minor synthetic products of species associated on morphological grounds.

## M. Biosynthesis of Aromatic Amino-acids

### (i) Tyrosine and phenylalanine

Quinic acid and shikimic acid (Fig. 28) have long been known as plant constituents but their biochemistry was neglected until recently. Quinic acid received some attention as a constituent, with caffeic acid,

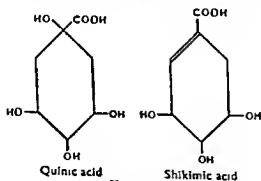


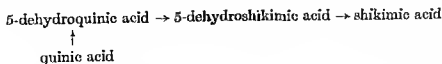
FIG. 28.

of chlorogenic acid, the main substrate for the polyphenol oxidase that causes browning in damaged tissues of apples and pears. Free quinic and shikimic acids are now recognized as normal constituents of many

plant tissues, and as intermediates in the synthesis of aromatic amino-acids by the micro-organisms (*Escherichia coli* and *Neurospora crassa*) with which this process has mainly been studied. Progress in this field followed the discovery of mutants in which the normal synthetic sequence was blocked at various points. These mutants accumulated, in amounts large enough for identification, different intermediates which in the normal organism were promptly used in further transformations and thus were inaccessible to study.

Shikimic acid replaces tyrosine and phenylalanine in mutants of *Escherichia coli* (Davis, 1951) and of *Neurospora* (Tatum, Gross, Ehrensward, & Garnjobst, 1954) which cannot form the aromatic amino-acids. Shigecura & Sprinson (1952) isolated shikimic acid from cultures of *E. coli* in which the synthesis was blocked at a later stage, and showed that labelled carbon supplied to the bacteria in shikimic acid appeared in tyrosine. These findings established shikimic acid with reasonable certainty as a precursor of the aromatic amino-acids. Further work with *E. coli* indicated two earlier intermediates, 5-dehydroshikimic acid and 5-dehydroquinic acid (Salamon & Davis, 1953; Weiss, Davis, & Mingioli, 1953).

The position of quinic acid in this sequence is less clear. Gordon, Haskins, & Mitchell (1950), finding it to be a growth factor for a *Neurospora* mutant, suggested that it was a precursor of the aromatic amino-acids. Davis & Weiss (1953) showed that mutants of *Aerobacter* using 5-dehydroquinic acid grew also with quinic acid. Quinic and shikimic acids are interconvertible in *Lactobacillus pastorianus* var. *quinicus* (Carr, Pollard, Whiting, & Williams, 1957). Other organisms, however, lack the enzyme reducing quinic acid to 5-dehydroquinic acid. Quinic acid is thus apparently off the main pathway, but can be a precursor of aromatic amino-acids in organisms converting it to 5-dehydroquinic acid. This part of the sequence may be represented:



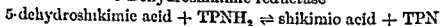
There is evidence (Carles & Lattes, 1953) that in germinating seedlings of wheat and lupin quinic acid is a catabolic product of phenylalanine and other aromatic compounds stored in the seed, and is further metabolized to malonic acid.

Some mutants of *Escherichia coli* convert shikimic acid to 5-phosphoshikimic acid (Weiss & Mingioli, 1956); it is not, however, certain

whether this is an obligatory intermediate in the sequence. Another unidentified metabolite of shikimic acid, known as  $Z_1$ , is accumulated by some mutants; it occurs later in the sequence than 5-phosphoshikimic acid (Davis & Mingioli, 1953) and is believed to be an intermediate between shikimic acid (or 5-phosphoshikimic acid) and the next definitely established member of the sequence, prephenic acid. This dicarboxylic acid apparently arises by a condensation of shikimic acid and pyruvic acid; it is very labile, decarboxylating in acid media to form phenylpyruvic acid (Weiss, Gilvarg, Mingioli, & Davis, 1954). At pH 7 its half-life at room temperature is 130 hours. Prephenic acid is a close precursor of phenylalanine, the amino analogue of phenylpyruvic acid. It is also a precursor of *p*-hydroxyphenyllactic acid (Ghosh, Adams, & Davis, 1956), which probably leads via *p*-hydroxyphenylpyruvic acid to its amino analogue, tyrosine. *p*-Hydroxyphenyllactic acid may, however, be a side-product rather than an intermediate in the sequence leading to tyrosine (Schwink & Adams, 1959). Prephenic acid accumulates in a mutant of *Neurospora crassa* unable to form aromatic amino-acids (Metzenberg & Mitchell, 1956).

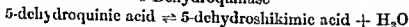
Enzymes catalysing the following stages have been prepared and partially purified:

5-Dehydroshikimic reductase



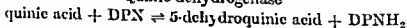
(from *Aerobacter aerogenes*, *E. coli*, yeast, peas, spinach leaves; Yaniv & Gilvarg, 1955).

5-Dehydroquinase



(*Aerobacter*, *E. coli*; Mitsuhashi & Davis, 1954).

Quinic dehydrogenase



(*Aerobacter*; Mitsuhashi & Davis, 1954).

In considering possible carbohydrate precursors for shikimic acid, which has seven carbon atoms, a heptose has obvious advantages. Bacterial extracts incorporated some labelled carbon into shikimic acid from sedoheptulose-7-phosphate, but the yield was only about 5 per cent, as with various hexose phosphates and diphosphates (Kalan, Davis, Srinivasan, & Sprinson, 1956). Sedoheptulose-1,7-diphosphate, on the other hand, was efficiently converted to shikimic acid (Srinivasan, Sprinson, Kalan, & Davis, 1956), but the contributions of different

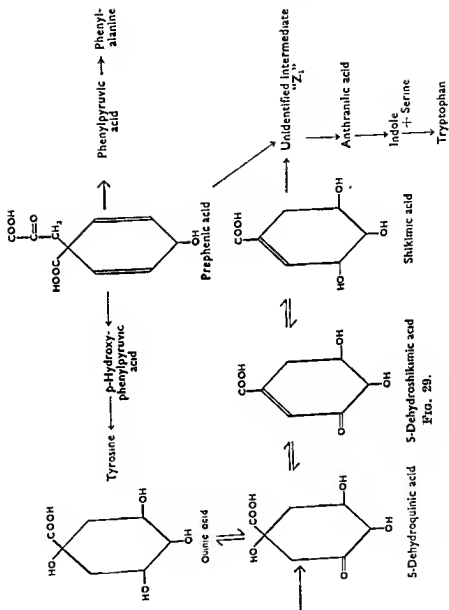
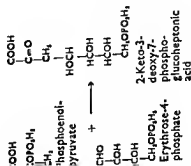


FIG. 29.



carbon atoms of the heptose to the molecule of shikimic acid, as established by labelling experiments, were inconsistent with its direct cyclization. The data favoured its cleavage to fragments with three and four carbon atoms, sedoheptulose was, moreover, completely replaceable as a precursor of shikimic acid by a mixture of phosphoenolpyruvate (3 carbon atoms) and erythrose phosphate (4 carbon atoms). The heptose diphosphate, although an excellent precursor for the aromatic amino acids, is thus not an obligatory intermediate in their formation.

Dihydroshikimic acid is converted in *Neurospora crassa* (Tatum, Gross, Ehrensverd, & Garnjobst, 1954, Gross, 1958), and in a variety of *Pseudomonas ovalis* (Hattori, Yoshida, & Hasegawa, 1958) to protocatechuic acid (3,4 dihydroxybenzoic acid), another simple aromatic compound. This is a more direct route to the aromatic ring than via prephenic acid. There is evidence (Shimazono, Schubert, & Nord, 1958) that the wood rotting fungus *Lentinus lepideus* synthesizes the aromatic compound methyl *p* methoxycinnamic acid from glucose via shikimic acid. The biosynthesis of aromatic amino acids in micro organisms is summarized in Fig. 29.

Studies with micro organisms have thus substantiated and extended the suggestions of Dangschat & Tischer (1938), who suggested, on mainly chemical grounds, the biosynthetic sequence

glucose  $\rightarrow$  quinic acid  $\rightarrow$  shikimic acid  $\rightarrow$  aromatic compounds

There is some evidence, apart from the widespread occurrence of quinic and shikimic acid, for these synthetic pathways in higher plants. Brown & Neish (1954, 1955) showed that in wheat (*Triticum vulgare*) and in maple (*Acer negundo* var. *interius*) phenylalanine labelled with  $C^{14}$  was an effective precursor of lignin, incorporation of labelled carbon from shikimic acid was as efficient as from phenylalanine. Acerbo, Schubert, & Nord (1958) supplied labelled *p* hydroxyphenylpyruvic acid to a growing sugar cane plant, and showed that it was incorporated as a unit, without disruption of the phenylpropane skeleton, into lignin. Nord and his co-workers also provided evidence that in sugar cane labelled shikimic acid was a precursor of lignin. It thus seems likely that the  $C_6-C_3$  (phenylpropane) structure of phenylalanine and tyrosine, which is also the unit structure of lignin, derives from shikimic acid in higher plants as in micro organisms. In ripening wheat ears supply of phenylpyruvic acid induced a very active synthesis of phenylalanine, the nitrogen used coming mainly from glutamic acid and



glutamine (Kretovich & Uspenskaya, 1959). Kretovich & Uspenskaya (1958) showed that glutamic acid transaminated with phenylpyruvic acid to form phenylalanine in homogenates of pea seedlings; other amino-acids tested were much less active donors of amino-groups. In wheat and another grass (*Calamagrostis inexpectans*) tyrosine was a precursor of lignin; it was inactive in eleven other species from ten families (Brown & Neish, 1956). Phenylalanine seems a more general precursor of lignin. The aromatic amino-acids would, of course, be decaminated before utilization of their carbon skeletons in lignin formation. Twigs of spruce (*Picea excelsa*) form lignin from labelled phenylalanine by the sequence shown in Fig. 30 (Freudenberg & Niedercorn, 1958).

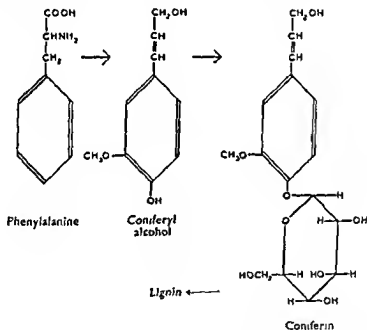
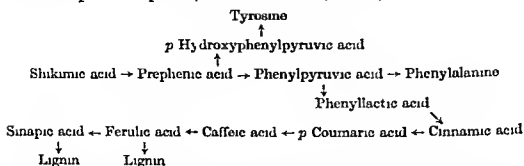


FIG. 30.

McCalla & Neish (1959a) showed that in *Salvia splendens* (Labiatae) shikimic acid labelled with  $\text{C}^{14}$  was an effective precursor of both phenylalanine and tyrosine. Quinic acid was converted into shikimic acid, phenylalanine, and tyrosine in rose cuttings (Weinstein, Porter, & Laurencot, 1959). In wheat (*Triticum*) and buckwheat (*Fagopyrum*) phenylalanine and its precursors (phenyllactic acid, phenylpyruvic acid) were hydroxylated to form tyrosine (Gamborg & Neish, 1959). McCalla & Neish (1959b) found phenylalanine (but not tyrosine) a good precursor of caffeic (3,4-dihydroxycinnamic), *p*-coumaric (4-

hydroxycinnamic), ferulic (3 methoxy-4 hydroxycinnamic) and sinapic (3,5-dimethoxy 4 hydroxycinnamic) acids. These acids all have the  $C_6-C_3$  carbon skeleton of phenylalanine, with a double bond in the  $C_3$  side chain. They are widely distributed among plants and appear to be precursors of lignin. The following scheme is suggested for their interrelationships in the plant (McCalla & Neish, 1959b)

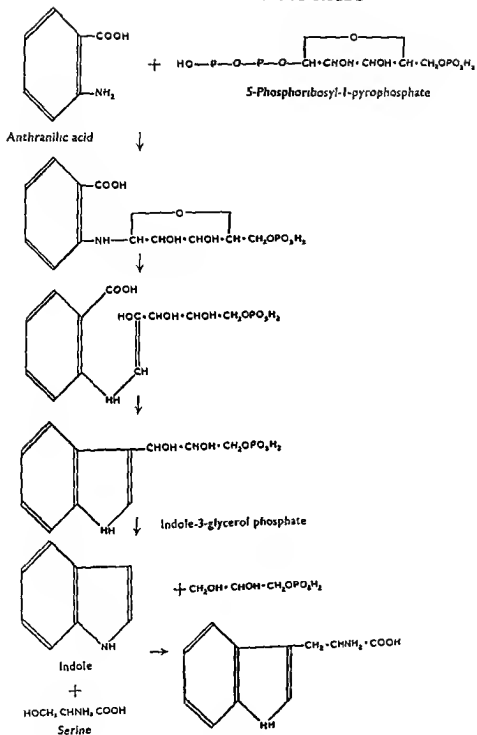


Lignin is generally considered to be a nitrogen free substance. Lignins from annual plants, however, contain 1-2 per cent of nitrogen, which is very tenaciously retained during purification and may be an integral part of the molecule. They yield amino acids on hydrolysis (Meyer & Bondi, 1952). Ter Karapetyan & Ogandzhanyan (1960) also found material yielding amino acids on hydrolysis to be firmly bound to lignin, cellulose, and hemicellulose from herbaceous plants. Whitehead & Quicke (1960) found that lignin from grasses contained nitrogen, partly in *N*-methyl groups, after repeated purification with dioxan.

The shikimic acid pathway is probably not the only biosynthetic route for aromatic compounds. The origin of the benzene ring from acetate units was considered by Collie (1907) and by Robinson (1955), the idea has experimental support for the synthesis of 6-methylsalicylic acid and of griseofulvin by *Penicillium griseofulvum* (Birch & Donovan, 1953; Birch, Massy Westropp, & Moye, 1955; Birch, Massy Westropp, Richards, & Smith, 1957). In buckwheat (*Fagopyrum*) one aromatic ring of quercetin appears to arise from shikimic acid and another from acetate (Underhill, Watkin, & Neish, 1957).

## (ii) Tryptophan

Fildes (1940) showed that bacteria formed tryptophan from indole. It was later established (Umbreit, Wood, & Gunsalus, 1946; Yanofsky, 1952) that in *Neurospora crassa* and *Escherichia coli* a phosphopyridoxal enzyme catalyses the condensation of indole and serine to tryptophan. There is also evidence that shikimic acid is a precursor of tryptophan, and so presumably of indole, in *E. coli* (Davis, 1951). Other compounds



Tryptophan  
 FIG. 31.  
 (Yanofsky, 1957)

used in tryptophan synthesis by some micro organisms include nicotinic acid (*Neurospora* Beadle, Mitchell, & Nye, 1947) and anthranilic acid (bacteria Snell, 1943, *Neurospora* Tatum, Bonner, & Beadle, 1944, *Neurospora* and *E coli* Yanovsky, 1955)

Yanovsky (1956a, b, 1957) clarified the intermediate stages between anthranilic acid and tryptophan. He prepared two protein fractions from extracts of *E coli*. Fraction A converted anthranilic acid, in the presence of magnesium ions and of 5 phosphoribosyl 1 pyrophosphate, to indolyl 3 glycerol phosphate, which fraction B converted to indole and triose phosphate (Fig. 31). The indole was then condensed with serine by tryptophan synthetase to form tryptophan. The available evidence suggests that this pathway occurs in *Salmonella typhimurium* (Brenner, 1955, Lingens & Hellmann, 1957) and in *Neurospora* (Tatum, Bonner, & Beadle, 1944) as well as in *E coli*. In *Saccharomyces* some other pathway appears to operate (Parks & Douglas, 1957). Indole may not be an intermediate in all species, as tryptophan could be formed from indolyl 3 glycerolphosphate without production of free indole. Anthranilic acid may arise *in vivo* from shikimic acid. It is formed from 5 phosphoshikimic acid and glutamine by an enzyme in cell free extracts of *Escherichia coli* (Srinivasan, 1959). Glutamine was much the most effective amino group donor tested, slight synthesis occurred also with asparagine, glutamic acid, and ammonium chloride.

The synthesis of tryptophan in higher plants remains little known. They produce numerous derivatives of anthranilic acid and of indole, the free compounds, recorded mainly from essential oils, may be artifacts arising by the breakdown of more complex precursors during processing. Polyanovski & Kretovich (1957) infiltrated shoots of pea seedlings with possible precursors of tryptophan and determined their tryptophan content 12 hours later. Considerable synthesis of tryptophan followed infiltration of serine plus indole or of serine plus anthranilic acid. Indole alone gave little synthesis and serine alone gave none. It thus appears that tryptophan is formed in the pea from serine and indole, the latter arising from anthranilic acid. The formation of indole derivatives from tyrosine has been demonstrated in studies of the formation of melanin mainly with animal material (Raper 1926, 1927, Beer, Clarke, Khorana & Robertson 1948a, b).

## N Biosynthesis of Histidine

The biosynthesis of histidine has been studied almost exclusively in micro-organisms. Studies with labelled metabolites indicate formic acid

(Levy & Coon, 1951), glucose, and acetic acid (Levy & Coon, 1954) as efficient precursors of individual carbon atoms of histidine. All these compounds must, however, require considerable transformation to produce the histidine molecule, or its earliest precursors containing the imidazole ring. Three precursors with this ring, accumulated by mutants of *Neurospora crassa* unable to synthesize histidine, were identified (Ames & Mitchell, 1955) as imidazoleglycerol phosphate, imidazoleacetol phosphate, and histidinol phosphate. These are shown

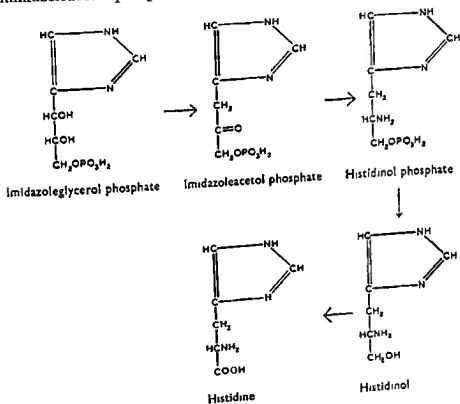


FIG. 32.

in Fig. 32 together with the synthetic sequence that seems probable in *Neurospora*. Data consistent with this pathway have also been obtained for *E. coli* (Westley & Ceithaml, 1956). It is uncertain whether histidinol or histidinol phosphate is the immediate precursor of histidine. Enzymes from yeast and *E. coli* oxidized histidinol (Ames & Mitchell, 1955). Enzymes catalysing early stages in the sequence of Fig. 32 are also known from *Neurospora*. Imidazoleglycerol phosphate dehydrogenase (Ames, 1957b) forms imidazoleacetol phosphate; it requires manganese ions. A transaminase (Ames & Horecker, 1956) then forms histidinol

phosphate, which is hydrolysed to histidinol by a specific phosphatase (Ames, 1957a).

The origin of the imidazole ring has been studied using mutants of *E. coli*. Guanine can supply the N—3 atom of the imidazole ring of histidine, together with an adjacent carbon atom (Magasanik, 1956); adenine is, however, a more efficient precursor (Moyed & Magasanik, 1957; Neidle & Waelsch, 1959). Glutamine is an efficient and apparently somewhat specific source of the N—1 atom; it is not replaceable by the amide group of asparagine, the amino groups of aspartic and glutamic acids, or ammonia (Neidle & Waelsch, 1959). The purines are replaceable by aspartic or glutamic acids as sources of the N—3 atom.

### O. Arginine, Citrulline, Ornithine, and the Urea Cycle

The formation of ornithine from glutamic acid has already been mentioned. In mammals (Krebs & Henseleit, 1932) and reptiles (Manderscheid, 1933) urea is formed from ammonia and carbon dioxide by a cyclic process involving ornithine, citrulline, and arginine (Fig. 33). Evidence of similar reactions was obtained by studies of mutants in

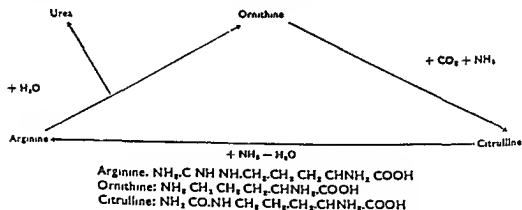
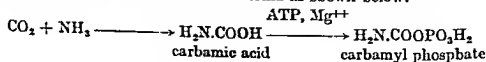


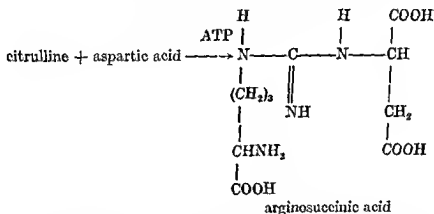
FIG. 33.

*Neurospora* (Srb & Horowitz, 1944; Fincham, 1953), *Penicillium* (Bonner, 1946), and *Aspergillus* (Pontecorvo, 1950). In *Streptococcus faecalis* (Jones, Spector, & Lipmann, 1955), *Serratia marcescens* (Glasziou, 1956), and mung bean mitochondria (Bone, 1959) carbamyl phosphate, an intermediate in the formation of citrulline from ornithine, arises from carbon dioxide and ammonia as shown below:



In animal tissues *N*-glutamyl derivatives such as *N*-carbamylglutamic acid, *N*-formylglutamic acid, or *N*-acetylglutamic acid are required (Grisolia & Cohen, 1953; Hall, Metzenberg, & Cohen, 1956), but their participation in the reaction has not been shown for micro-organisms.

The formation of arginine from citrulline has also been separated into two enzymatic stages. Citrulline and aspartic acid condense in the presence of adenosine triphosphate to form arginosuccinic acid, which is then split to form arginine and fumaric acid (Ratner, Petrack, & Roehovansky, 1953). The same compound is formed from arginine and fumaric acid by enzymes from peas and lupin seeds (Davison & Elliott, 1952) and from *Chlorella pyrenoidosa* and seeds of *Canavalia ensiformis* (Walker & Myers, 1953). A similar condensation of fumaric acid with canavanine, catalysed by enzymes from *C. ensiformis* and from various micro-organisms, produces canavanosuccinic acid (Walker, 1953). The reactions involved may be summarized as follows:



The condensation of citrulline with aspartic acid is the major pathway of urea formation in the liver, other amino-acids being converted to aspartic acid by transamination (Kluge, 1956; Braunstein, 1957). The key position of aspartic acid in this process is shown by the suppression of urea synthesis when  $\alpha$ -methylaspartic acid is added to liver preparations. This substance, an antimetabolite of aspartic acid, specifically inhibits its condensation with citrulline to form arginosuccinic acid. It does not affect other reactions of the ornithine cycle (Braunstein, Severina, & Babskaya, 1956). The conclusion that aspartic acid is a major precursor of urea in mammals was also reached by Von Knierem (1874), on rather slender evidence from feeding tests with intact animals.

There are some indications that formation of urea in the liver is

more complex than the Krebs Henseleit cycle indicates Gornall & Hunter (1943) showed that ornithine was more effective than citrulline as a catalyst of urea synthesis in rat liver. This was confirmed by Bronk & Fisher (1956) who proposed a combination of two cycles, each involving hypothetical derivatives of ornithine and citrulline. Della Pietra, Roghani, Roghani, & Andreucci (1959) found that preparations from rat liver formed urea from carbamylaspartic acid with ornithine, but not with citrulline unless adenosine triphosphate was added. All these observations are hard to interpret on the basis of the simple ornithine cycle, but seem to require its modification rather than its abandonment.

Arginase, which catalyses the breakdown of arginine to ornithine and urea, is known from animals (Kossel & Dakin, 1904), yeast (Shiga, 1904, Edlbacher, Becker, & Scgesser, 1938), higher plants (e.g. *Angelica sylvestris*, *Trifolium pratense*) (Kiesel, 1911, 1922a) and higher fungi (Yamamoto, Ertate, & Miwa, 1953). It occurs in *Canavalia ensiformis* (Damodaran & Narayanan, 1940), *Atropa belladonna* (James, 1949), *Dolichus lablab* (Vaidyanathan & Giri, 1953), and *Pinus pinaster* (Guitton, 1959). Fries (1953) showed that ornithine or citrulline satisfied the arginine requirement of excised pea roots. There is evidence that the ornithine cycle occurs in soybean leaves (Racusen & Aronoff, 1954), groundsel (*Senecio vulgaris*) roots (Skinner & Street, 1954) and seedlings of watermelon (*Citrullus vulgaris*) (Kasting & Delwiche, 1955) and pea (Reifer & Buraczewski, 1958).

## P. Synthesis of Lysine

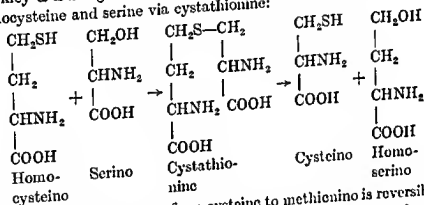
The biosynthesis of lysine is not fully understood in any organism, particularly little is known about it in higher plants. Complex interrelations exist or are suspected between lysine and other straight-chain or cyclic acids with 6 carbon atoms, including  $\alpha$  keto adipic acid,  $\alpha$  amino adipic acid,  $\alpha$  keto  $\epsilon$  aminocaproic acid,  $\epsilon$  hydroxy  $\alpha$  aminocaproic acid, pipercolic acid and  $\Delta^1$  piperidine 2 carboxylic acid. Lysine arises in some bacteria by decarboxylation of  $\alpha$   $\epsilon$  diaminopimelic acid (Dewey, Hoare, & Work, 1954) but the known distribution of this amino acid is limited and it seems unlikely to provide a general pathway to lysine. Davis (1952) showed that it could replace lysine for some mutants of *Escherichia coli* in which it may be formed by a condensation of aspartic acid with pyruvic acid (Abelson, Bolton, Britten, Cowie, & Roberts, 1953). Acetate and succinate seem to be precursors of  $\alpha$



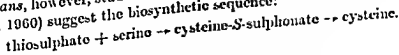
keto adipic acid and lysine in *Torulopsis utilis* (Strassman & Weinhouse, 1953).

### Q. Synthesis of Sulphur-containing Amino-acids

The metabolism of these amino-acids has been studied in mammals and in micro-organisms, particularly *Neurospora crassa*; little direct information is available for higher plants. Horowitz (1947) studied four strains of *N. crassa*, which had lost, by single-gene mutations, the ability to synthesize methionine. One strain used cysteine, cystathionine, and homocysteine; another cystathionine and homocysteine; the third homocysteine; the fourth methionine only. This, plus supporting evidence such as accumulation of cystathionine by a strain that could not use it, suggested for the normal organism the synthetic sequence: cysteine  $\rightarrow$  cystathionine  $\rightarrow$  homocysteine  $\rightarrow$  methionine. In the rat (Binkley & Du Vigneaud, 1942; Stetten, 1942) cysteine is formed from homocysteine and serine via cystathionine:



The cystathionine pathway from cysteine to methionine is reversible in *Neurospora*. Methionine is demethylated to homocysteine, which reacts with serine to form cystathionine, and thus cysteine and homoserine. Pyridoxal phosphate takes part in these reactions (Braunstein & Goryachenkova, 1950). Folic acid co-enzymes are involved in the synthesis of methionine from serine and homocysteine by extracts of *Escherichia coli* (Szulmajster & Woods, 1960). The actual introduction of sulphur into the amino-acid molecules is not clearly understood. It enters the plant as sulphate, which is reduced, probably via sulfito and thiosulphate, to the sulphydryl reduction level before combining with serine or homoserine to form the corresponding sulphur-containing amino-acids. Hydrogen sulphide may be involved. In *Aspergillus nidulans*, however, studies on mutants (Hockenull, 1949; Nakamura & Sato, 1960) suggest the biosynthetic sequence:



## CHAPTER 9

# THE BREAKDOWN OF AMINO-ACIDS

### A. General

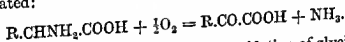
Several pathways of breakdown exist in plants, some are available to all or most amino acids, others to a few only. These catabolic pathways have been more thoroughly studied in animal tissues and in micro-organisms than in plants. Much evidence cited in this section therefore comes from organisms other than higher plants. It is relevant here because where comparative data are available the mechanisms of breakdown in higher plants resemble those of other organisms. This general similarity does not, however, exclude particular differences, and we cannot assume that metabolic sequences established for one organism necessarily occur in another.

### B. Oxidation by Polyphenol Oxidase Systems

The first polyphenol oxidase to be studied was found (Yoshida, 1883) in the latex of the lac tree (*Rhus vernicifera*, Anacardiaceae), it initiates the complex series of changes transforming this latex to the hard shining black pigment used in Chinese and Japanese lacquer work. Enzymes of this type were first called laccases and later tyrosinases, neither being particularly appropriate, polyphenol oxidase is now generally used. Bertrand (1894, 1895a, b) prepared oxidizing enzymes with a wide range of substrates among aromatic compounds with a hydroxyl or amino group. He found enzymes of this type in various organs of many plants, including *Rhus succedanea* (another lac tree), beetroot, apple, asparagus, canna, carrot, clover, dahlia, lucerne (alfalfa), pear, potato, quince, turnip, and others, though they appeared to be absent from some species. Purified polyphenol oxidases from potato (Kubowitz, 1937, 1938), mushroom (*Psalliota campestris*) (Keilin & Mann, 1938) and *Rhus succedanea* (Keilin & Mann, 1939) are all copper proteins.

In some tissues, e.g. carrot root (Marsh & Goddard, 1939), spinach leaves (Bonner & Wildman, 1946) and leaves of the tea plant (*Camellia sinensis*) (Sreenagachar, 1943; Li & Bonner, 1947; Bokuchava, 1946, 1948; Roberts & Wood, 1950), polyphenol oxidases are important

terminal oxidases in respiration, the quinones formed by oxidation of natural polyphenols acting as hydrogen acceptors. These quinones also oxidize amino-acids. Oxidative deamination of amino-acids by polyphenol oxidases, was demonstrated for enzymes of animal origin by Happold & Raper (1925), and for fungal enzymes by Robinson & McCance (1925). The actual deamination is probably non-enzymatic, as shown for the deamination of glycine by chlorogenic acid (Oparin, 1927). A polyphenol oxidase from *Atropa belladonna*, in the presence of a suitable substrate such as catechol, oxidized glycine, alanine, and ornithine to glyoxylic acid, pyruvic acid, and  $\alpha$ -keto- $\delta$ -aminovaleric acid. Other amino-acids were oxidized, but too slowly to permit isolation of the corresponding keto-acids (Beever & James, 1948; James, Roberts, Beever, & De Kock, 1948). The overall relation may be formulated:



Trautner & Roberts (1950) studied the oxidation of glycine *in vitro* by catechol-polyphenol oxidase systems from *Atropa belladonna* and *Duboisia myoporoides*. They considered a highly coloured pigment, formed by condensation in equimolecular proportions of o-quinone and an amino-acid, to be the actual oxidant, and proposed a cycle sequence of reactions regenerating the o-quinonoid pigment and so producing ammonia continuously from amino-acids. Hubbard (1938) put forward a somewhat similar but less detailed scheme. Popov (1956) studied the oxidation of amino-acids during "fermentation" of tea leaves. (It may be noted that in the processing of tea leaves, the dominant changes are due to enzymes of the leaf itself, not to micro-organisms. The same is probably true of the "fermentation" in tobacco processing. The traditional term is thus misleading, but is unlikely to be superseded.) Popov (1956) found that in the presence of polyphenol oxidase and the tannins of the tea leaf, amino-acids were oxidized to the corresponding aldehydes with liberation of carbon dioxide and ammonia. He suggested that amino-acids were oxidized by a quinone formed by polyphenol oxidase from epicatechin, a complex catechol derivative found in the tea leaf. Glycine was the most readily oxidized amino-acid, followed by alanine, phenylalanine, and valine. The aldehydes produced contribute to the flavour of tea brewed from fermented leaves ("black" tea).

The place of tyrosine (which is both a monophenol and an amino-acid) in these reactions is somewhat obscure. In animal systems it is oxidized to dihydroxyphenylalanine, which leads to 5,6-dihydroxyin-

dole 2 carboxylic acid, 5,6 dihydroxyindole, and indole 5,6 quinone, the last of these polymerizes to produce the black pigment melanin (Raper, 1926, 1927, Beer, Clarke, Khorana, & Robertson, 1948b). Tyrosine is a precursor of dark pigments in the pod of *Vicia faba* (Bourquelot & Herissey, 1898) and in injured tubers of potato (Haehn, 1919, Onslow, 1919, Schmalfuss & Buinbacher, 1943) and dahlia (Bertrand, 1896a, b). Boswell (1945) found, however, that potato polyphenol oxidase oxidized tyrosine only slowly, and that the enzyme-tyrosine system did not deaminate glycine. Enzyme diphenol systems from potatoes oxidized glycine and other amino acids. Steward, Berry, Preston, & Ramamurti (1943) also considered the phenolase system of potato tubers to be involved in deamination of amino acids. 3,4 Dihydroxyphenylethylamine (hydroxytyramine) is a substrate for polyphenoloxidase in fruits of banana (*Musa* sp.) (Griffiths, 1959), and probably of broom (*Sarothamnus scoparius*) (Schmalfuss, Barthmeyer, & Brandes, 1927). Tyrosine residues in proteins can be oxidized *in situ* to dopaquinone residues (Lissitzky, Rolland, & Lasry, 1960).

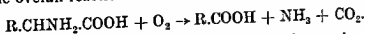
Polyphenol oxidases, or rather the quinones produced by their action on various natural substrates, are efficient oxidants for some, but not all, amino acids. Their importance *in vivo*, and their relation to metabolic processes utilizing the ammonia produced, can hardly be assessed on the information now available.

Rubin & Ivanova (1958) compared the oxidation of amino acids in the cabbage variety Anager, which is resistant to *Bolrytis cinerea*, and in the variety Number One, which is non resistant to this fungus. The resistant variety had a much higher content of almost all the amino acids studied, and also a more active amino acid oxidation after infection. The authors attribute a protective role to the oxidizing system.

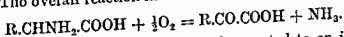
### C General Amino-acid Oxidases

Enzymes oxidizing a wide range of amino acids occur sporadically in animals, bacteria, and fungi but are not known from higher plants. A soluble enzyme from *Neurospora crassa* (Bender & Krebs, 1950, Thayer & Horowitz, 1951, Burton 1951) was very active towards alanine,  $\alpha$  aminobutyric acid,  $\alpha$  aminovaleric acid,  $\alpha$  aminocaproic acid,  $\alpha$  aminoadipic acid,  $\alpha$  aminopimelic acid, leucine, methionine, cystine, ornithine, histidine and phenylalanine, fairly active towards arginine, citrulline, canavanine, glutamine, glycine, serine, valine, isoleucine, tyrosine, tryptophan, lysine and glutamic acid, slightly active towards aspartic acid and threonine and inactive towards proline. The pro-

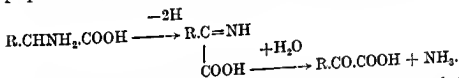
thetic group of the enzyme is flavin adenine dinucleotide (Burton, 1951); the overall reaction which it catalyses is:



Keto-acids are first formed, a general initial step in oxidative deamination (Neubauer, 1909; Knoop, 1910), but are oxidized by hydrogen peroxide formed by reaction of oxygen with flavin adenine dinucleotide. Knight (1948) obtained from *Aspergillus niger* and various species of *Penicillium* an insoluble enzyme oxidizing several amino-acids to the corresponding keto-acids, which were not further oxidized because catalase present in the preparations removed any hydrogen peroxide. The overall reaction involved is:



The amino-acid is probably first dehydrogenated to an imino-acid, which hydrolyses non-enzymatically to a keto-acid, as in animal preparations (Krebs, 1933; Euler, Adler, Gunther & Das, 1938):



Mycelia of *Fusarium culmorum* deaminate methionine to  $\alpha$ -keto- $\gamma$ -methylthiolbutyric acid (Tolba & Saleh, 1950), which could arise either by the action of an L-amino-acid oxidase or by transamination.

L-amino-acid oxidases have been obtained from *Aerobacter aerogenes*, *Proteus vulgaris*, and *Pseudomonas pyocyaneus* (Stumpf & Green, 1944), and from *Clostridium saccharobutyricum* and *C. sporogenes* (Rosenberg & Nisman, 1949). General D-amino-acid oxidases occur in some moulds (Horowitz, 1944; Emerson, Puziss, & Knight, 1950); some bacteria oxidize D-amino-acids (Bernheim, Bernheim, & Webster, 1935; Webster & Bernheim, 1936), but their range of substrates seems smaller than in the moulds. The specificity of the general amino-acid oxidases is uncertain; some workers consider that single enzymes in this group have very wide substrate ranges; others, e.g. Edlbacher & Grauer (1944), Stumpf & Green (1944), and Still, Buell, Knox, & Green (1949), hold that individual enzymes exist for some at least of the amino-acids.

Homogenates of rye (*Secale cereale*) and of pea (*Pisum sativum*) oxidize a wide range of amino-acids, atmospheric oxygen being consumed (Kretovich & Drozdova, 1948; Kretovich & Uspenskaya, 1952).

It is not clear whether the preparations contain a single enzyme of low specificity, or more numerous enzymes specific for individual amino acids. Oxidation of some amino acids may be indirect, the carbon chain being broken down after loss of the amino group by transamination. Both rye and pea preparations oxidized glutamic and aspartic acids more actively than any other amino acid tested. In each case oxidation of glutamic acid was more active than that of aspartic acid. The Russian authors found the same preferential oxidation of aspartic and particularly glutamic acid by polyphenol oxidase in seedlings of sunflower (*Helianthus annuus*).

### D Decarboxylation

The amino acids known to be decarboxylated *in vivo* are listed in Table 7, together with the products of the reaction. These products are amines, except when one carboxyl group only of a dicarboxylic amino acid is attacked, forming a non  $\alpha$  amino acid. Most of the amines were first recognized as products of bacterial breakdown of protein. Gale and his associates made a wide survey of amino acid decarboxylation by bacteria, and studied intensively some of the enzymes involved (Gale

TABLE 7

*Amino acids and their naturally occurring decarboxylation products*

<i>Amino-acid</i>	<i>Decarboxylation product</i>	<i>References</i>
Valine	Isobutylamine	Neuberg & Karczag (1930), King (1953)
Isoleucine	$\beta$ Methylbutylamine	Proom & Weiswed (1950)
Leucine	Isoamylamine	Arai (1921), King (1953)
Lysine	Cadaverine	Ladenberg (1886), Gale & Laps (1944), Ambo & Sohoni (1950)
Ornithine	Putrescine	Von Udránsky & Baumann (1888), Taylor & Gale (1945)
Arginine	Agmatine	Gale (1940a), Taylor & Gale (1945); Ambo & Sohoni (1950)
1 Phenylalanine	$\beta$ 1 Phenylethylamine	Jeanneret (1877), Gautier & Etard (1882), Fumagalli (1897)

TABLE 7 (Continued)

*Amino-acids and their naturally occurring decarboxylation products*

<i>Amino-acid</i>	<i>Decarboxylation product</i>	<i>References</i>
Tyrosine	Tyramine	Cautier & Mourgues (1888); Ackermann (1909); Barger & Walpole (1909); Gale (1940b); Epps (1944)
3,4-Dihydroxyphenylalanine	Hydroxytyramine (3,4-Dihydroxyphenylethylamine)	Schmalfuss & Heider (1931); Epps (1944); Griffiths (1959); Ambe & Schonie (1959)
Histidine	Histamine	Ackermann (1910); Berthelot & Bertrand (1912a); Epps (1945); Ambe & Schonie (1959)
Glutamic acid	$\gamma$ -Aminobutyric acid	Abderhalden, Fromme, & Hirsch (1913); Okunuki (1939); Schales, Mims, & Schales (1946)
$\gamma$ -Methyleneglutamic acid	$\gamma$ -Amino- $\alpha$ -methylenebutyric acid	Fowden & Done (1953)
Aspartic acid	$\beta$ -Alanine	Ackermann (1911); Virtanen & Laine (1937); Ambe & Schonie (1959)
Tryptophan	Tryptamine	Berthelot & Bertrand (1912b); Gale (1946); Weissbach <i>et al.</i> (1959); Mitoma & Udenfriend (1960)
Diaminopimelic acid	Lysine	Dewey, Hoare, & Work (1954)
Serine	Aminoethanol	Nord (1919); Stetten (1942)
Glycine	Methylamine	Schmidt (1875); Emmerling (1897); Emmerling & Reiser (1902); Klein & Steiner (1928)
Alanine	Ethylamine	Hesse (1857); Stein von Kamienski (1957a)
$\alpha$ -Aminobutyric acid, $\gamma$ -aminobutyric acid	Propylamine	Stein von Kamienski (1957b)

TABLE 8

*Occurrence of decarboxylation products of amino acids*

<i>Decarboxylation product</i>	<i>Species from which recorded</i>	<i>References</i>
$\gamma$ Aminobutyric acid	Widespread	Dent <i>et al</i> (1947), Westall (1950)
$\beta$ Alanine	Widespread	Hulme & Arthungton (1950), Steward <i>et al</i> (1951)
$\gamma$ Amino $\alpha$ methylene butyric acid	<i>Arachis hypogaea</i>	Fowden & Dene (1953)
Isoamylamine	Widespread	Klein & Steiner (1928), Stein von Kamienska (1957a)
Isobutylamine	<i>Berberis vulgaris</i> , <i>Mahonia aquifolium</i> , <i>Rosa</i> sp., <i>Viburnum lantana</i> , 5 species of <i>Araceae</i> , 6 species of <i>Crataegus</i>	Klein & Steiner (1928), Stein von Kamienska (1957a)
Cadaverine	<i>Solanum tuberosum</i> , <i>Pisum sativum</i>	Yoshimura (1934), Miettinen (1955)
Putrescine	<i>Datura stramonium</i> , <i>Atropa belladonna</i> , <i>Citrus</i> spp. <i>Pisum sativum</i>	Ciamcian & Ravenna (1911), Goris & Larssonneau (1921), Hiwatari (1927), Cromwell (1943b), Herbst & Snell (1948), Miettinen (1955)
Agmatine	<i>Ambrosia artemisiifolia</i> , <i>Ricinus communis</i> <i>Secale cereale</i> <i>Pisum sativum</i>	Heyl (1919), Kiesel (1924b), Mourgue <i>et al</i> (1953), Miettinen (1955)
Histamine	<i>Urtica urens</i> , several species of <i>Chono podiaceae</i>	Emmelin & Feldberg (1947), Werle & Raub (1948)
Tyramine	<i>Sarothamnus scoparius</i> <i>Hordeum sativum</i> , <i>Crinum yuccaeiflorum</i> several species of <i>Loranthaceae</i>	Crawford & Watanabe (1914, 1916), Schmalfuss & Heider (1931), Erspamer & Falconieri (1952), Correale & Cortese (1953), Fowden & Dene (1954)



TABLE 8 (Continued)

Occurrence of decarboxylation products of amino-acids

Decarboxylation product	Species from which recorded	References
Hydroxytyramine	<i>Sarothamnus scoparius</i> , <i>Musa sapientum</i>	Schmalfuss & Heider (1931); Correale & Cortese (1953); Griffiths (1959)
Tryptamine	<i>Acacia floribunda</i> , <i>A. longifolia</i> , <i>A. pruinosa</i>	White (1944)
5-Hydroxytryptamine	<i>Ananas comosus</i> ; <i>Gossypium hirsutum</i> ; <i>Symplocarpus foetidus</i> ; <i>Mucuna pruriens</i> ; <i>Musa sapientum</i>	Bruce (1960); Bowden <i>et al.</i> (1954); Bulard & Léopold (1958); Wnalkes <i>et al.</i> (1958); Cartier <i>et al.</i> (1958)
Aminoethanol (ethanolamine)	<i>Crataegus</i> sp., <i>Pinus sylvestris</i> , <i>Pisum sativum</i> , various higher fungi (No record completely certain; derivatives of the base are widespread)	Kiesel (1922c); Hyde (1953); Neu & Fiedler (1954); Possingham (1956); Stein von Kamienski (1957b)
Methylamine	<i>Mercurialis annua</i> , <i>M. perennis</i> , numerous other species	Schmidt (1875); Cromwell (1949); Stein von Kamienski (1957a)
Ethylamine	<i>Bryonia dioica</i> , <i>Arum italicum</i> , <i>A. maculatum</i>	Stein von Kamienski (1957a)
$\beta$ -Phenylethylamine	<i>Crataegus</i> (8 spp.), <i>Pyrus communis</i> , <i>Cornus sanguinea</i> , <i>Vincetoxicum officinale</i>	Stein von Kamienski (1957a)
Propylamine	<i>Claviceps purpurea</i> (ergot)	Stein von Kamienski (1957b)

1946). Decarboxylases for arginine, histidine, lysine, ornithine, tyrosine, and glutamic acid were highly specific; the lysine enzyme also attacked hydroxylysine, the tyrosine enzyme attacked dihydroxyphenylalanine, and the glutamic acid enzyme attacked  $\beta$ -hydroxyglutamic acid. The molecule of the regular substrate was thus still accessible to the enzyme after insertion of a hydroxyl group. Pyridoxal phosphate is the prosthetic group of some, and possibly all, of these enzymes. Other workers have added to the list of bacterial decarboxylases, but it still lacks

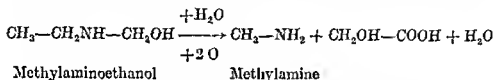
enzymes for many common amino-acids. Glycine and alanine, for instance, are not known to be decarboxylated. Their expected decarboxylation products, methylamine and ethylamine, occur in some higher plants; the latter appears to be a rare constituent; either may arise by processes other than decarboxylation. Threonine appears to be decarboxylated in *Streptomyces griseus*, where it is a precursor of the aminopropanol part of the molecule of vitamin B<sub>12</sub> (Krasna, Rosenblum, & Sprinson, 1957). Table 8 shows some occurrences of decarboxylation products in plants.

The only products of amino-acid decarboxylation known to occur widely in higher plants are  $\gamma$ -aminobutyric acid,  $\beta$ -alanine, and isoamylamine;  $\gamma$ -aminobutyric acid alone is produced by a widely distributed decarboxylase. Mazelis (1959) obtained a methionine decarboxylase from cabbage leaves; the decarboxylation product was not identified. Werle & Raub (1948) found histamine in several higher plants, including *Chenopodium bonus-henricus* and *Spinacea oleracea*. The flowers had the highest concentration of the amine and seeds very little. Appel & Werle (1959) confirmed the occurrence of histamine in *Spinacea oleracea*, finding also *N*-acetylhistamine, *N,N*-dimethylhistamine and traces of trimethylhistamine. Formation of histamine was attributed to decarboxylation of histidine. Seedlings of *Sarothamnus scoparius* (broom) decarboxylated dihydroxyphenylalanine to hydroxytyramine, recorded in this species by Schmalfuss & Heider (1931). Although intact spinach seedlings decarboxylated histidine their aqueous extracts and homogenates failed to catalyse the reaction. Grassmann & Bayerle (1934) obtained no decarboxylation of amino-acids by preparations from amine-producing flowers of various species. Similar negative results were reported for flowers of *Crataegus secunda*, *C. monogyna*, and sclerotia of *Claviceps purpurea* (Stein von Kamienski, 1957b). The chemically attractive theory of amine formation by decarboxylation of amino-acids has thus received very little experimental support in higher plants. The observation (Werle & Raub, 1948) that intact plants carry out a decarboxylation not duplicated in extracts suggests that further work is necessary before the idea can be regarded as definitely disproved.

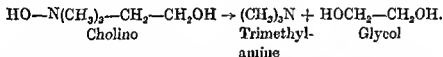
Ambe & Solonio (1959) studied the decarboxylation of aspartic acid, arginine, histidine, lysine, tyrosine, and dihydroxyphenylalanine by aqueous extracts from seeds of the legumes *Cajanus indicus*, *Cicer arietinum*, *Dolichos lablab*, *Lens esculentum*, *Pisum arvense*, *P. sativum*, *Phaseolus aconitifolius*, *P. aureus*, *Vicia faba*, and *Vigna catjang*.

Enzymes producing carbon dioxide from these amino-acids were widespread among the species tested. Other products of decarboxylation were not identified.

Stein von Kamienski (1957a) used an improved technique to study the distribution of amines in 220 species of flowering plants; 75 contained isoamylamine, 25 methylamine, 19 trimethylamine, 16  $\beta$ -phenylethylamine, 15 isobutylamine, 3 (*Arum italicum*, *A. maculatum*, *Bryonia dioica*) ethylamine, and one (*Heracleum sphondylium*) dimethylamine. Methylamine is apparently widespread in traces. In *Mercurialis perennis* it arises (Cromwell, 1949) from methylaminoethanol, an intermediate in the biosynthesis of choline:



Stein von Kamienski (1957b) suggested that methylamine and dimethylamine may arise by the action of mono-amine oxidases on trimethylamine; the last compound is formed from choline by bacteria (Cohen, Nisman, & Raynaud, 1947) and by an enzyme of *Chenopodium vulvaria* (Cromwell, 1950):



The enzyme could not be found in *Chenopodium album* (Cromwell, 1950). Methylamine has occasionally been recorded as a product of protein breakdown by bacteria, e.g. *Streptococcus longus* (Emmerling, 1897) and *Bacillus fluorescens liquefaciens* (Emmerling & Reiser, 1902).

Ethylamine, though arising by decarboxylation of a widespread amino-acid (alanine), is rare as a natural product. The only flowering plants known to produce it seem to be three species mentioned above, *Orataegus oxyacantha* (Neu & Fiedler, 1954), and *Sambucus nigra* (Steiner & Stein von Kamienski, 1953). There are old reports (Hesse, 1857; Muller, 1857; Sullivan, 1857) that it is formed in protein decomposition. It occurs (Honegger & Honegger, 1960) in mammalian brain. *Pseudomonas aeruginosa* produces ethylamine when grown with alanine,  $\beta$ -alanine, or D-phenylalanine as the sole source of nitrogen. It is not found in cultures supplied with L-phenylalanine. *Salmonella paratyphi* B forms it from alanine but not from L- or D-phenylalanine, this organism is unable to use  $\beta$ -alanine (Césaire, Neuzil, & Boiron,

1938a, b) Stein von Kamienski (1937b) found ethylamino in sterile and non sterile autolysates of the fruiting bodies of higher fungi (*Boletus*, *Russula*), and in sclerotia of ergot (*Claviceps purpurea*), which contain a wide range of amines methyl, trimethyl, ethyl, propyl, isopropyl, isobutyl, isoamyl, hexyl, and  $\beta$  phenylethyl. A similar though not quite identical group of amines is present in fruiting bodies of the higher fungus *Polyporus sulphureus* (List, 1958). Propylamine could arise by decarboxylation of either  $\alpha$  or  $\gamma$  aminobutyric acid, both occur free in ergot (Gröger & Mothes, 1956). Isopropylamine and hexylamino cannot be derived in this way from amino acids known in natural products.

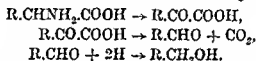
The production of small amounts of volatile amines, especially in the flowers, is characteristic of some plant families (Klein & Steiner, 1928, Steiner & Löffler, 1931, Stein von Kamienski, 1957a). Amines, for instance, are common in members of the Araccae, Caprifoliaceae, Cornaceae, and Rosaceae, they are absent from all investigated species of Labiatae and of the sub family Papilionatae of Leguminosae. Their occurrence among related species is erratic, in *Crataegus* three species each contained four different amines, four species each had three amines, four species had two amines, and in two species no amines were detected (Stein von Kamienski, 1957a).

The amines are oxidatively deaminated to the corresponding aldehydes by mono amino oxidases found in several higher plants (Werlo & Roewer, 1952), or by di amino oxidases also known from several species (Cromwell, 1943b, Hasse & Maisack, 1955, Mann & Smithies, 1955). The di amines yield on oxidation amino aldehydes which cyclize readily and *in vitro* lead to simple alkaloids (Hasso & Berg, 1957, Clarke & Mann, 1959, Mothes, Schutte Simon, & Weygand, 1959).

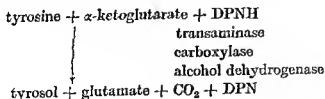
The deamination of amino acids in fermenting systems forms alcohols. Many alcoholic drinks contain, besides ethyl alcohol, small amounts of higher alcohols known collectively as fusel oil, these alcohols, and particularly their esters, are of some importance as flavouring substances. Muller (1857) suggested that amyl alcohol and amylamine, found in autolysing (or perhaps putrefying) beer yeast arose from leucine, and so from protein. They were identified on rather flimsy evidence, especially for the alcohol and their relation to leucine was not fully understood. Its mere recognition at this date is, however, noteworthy.

Lhrlich (1906, 1907, 1911, 1912) showed that isohutanol, isoamyl alcohol, tryptophol ( $\beta$  indoleethyl alcohol) and tyrosol arose by the action of yeast on valine, leucine, tryptophan and tyrosine present in

the fermenting material. He also found that the mould *Oidium lactis* and the yeast *Willia onomola* gave high yields of tyrosol when supplied with tyrosine (Ehrlich & Pistschimuka, 1912). Kurono (1909a) studied the formation of fusel oil in saké fermentation, and confirmed the production of amyl alcohol from leucine. Neubauer & Fromherz (1911) made detailed studies of the formation of benzyl alcohol from phenylglycine during fermentation. They established, in agreement with Ehrlich, that free ammonia did not appear, and that the process took place only during the fermentation of glucose. Phenylglyoxylic acid and benzaldehyde were recognized as intermediates, the following sequence of reactions being proposed for the formation of higher alcohols:



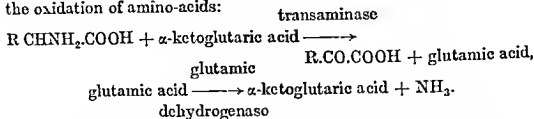
Leucine, isoleucine, and valine from yeast protein probably contribute to the formation of fusel oil if the medium is deficient in these amino-acids (Ehrlich, 1906; Castor & Guymon, 1952). Sentheshanmuganathan & Elsdon (1958) confirmed earlier observations that the formation of tyrosol from tyrosine by *Saccharomyces cerevisiae* is anaerobic and requires a supply of glucose. Cell-free extracts of the yeast formed glutamic acid, *p*-hydroxyphenylacetaldehyde, and carbon dioxide from tyrosine and  $\alpha$ -ketoglutaric acid, the reaction being stimulated by pyridoxal phosphato. Cell-free extracts also decarboxylated *p*-hydroxyphenylpyruvic acid and reduced the aldehyde so formed. Conversion of amino-acid to alcohol involves successively transamination, decarboxylation, and enzymatic reduction. The overall reaction is formulated as:



The function of glucose in the reaction is to supply reduced diphosphopyridine nucleotide by glycolysis and to provide  $\alpha$ -ketoglutaric acid for the initial transamination.

Transamination is often an early stage in the breakdown of amino-acids, as in the oxidation by animal tissues of tyrosine (Knox & Knox, 1951; Schepartz, 1951) and of tryptophan (Dalglish, Knox, & Neuberger, 1951; Wiss, 1952). These examples support the suggestion (Braun-

stein & Bychkov, 1939, 1940; Braunstein & Azarkh, 1945) that transamination to form glutamic acid, which is then deaminated by the highly specific glutamic dehydrogenase, may be a general pathway in the oxidation of amino-acids:

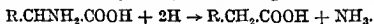


This scheme was originally based largely on evidence from animal material; the wider distribution in plants of glutamic dehydrogenase than of amino-acid oxidases suggests that the mechanism involved may be important in them also.

## E. Miscellaneous Pathways of Amino-acid breakdown

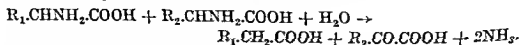
### (i) Reductive deamination

Reduction of aspartic acid by *Escherichia coli* (Harden, 1901) and of glycine, ornithine, and tryptophan by *Clostridium sporogenes* (Hoogerhede & Kocholaty, 1938) follows the general reaction:



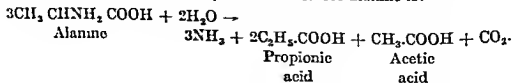
### (ii) The Stickland reaction and other dismutations

This reaction, named after its first investigator, is mediated by *Clostridium sporogenes* (Stickland, 1934, 1935a, b, c; Woods, 1936). Two amino-acids interact according to the equation below, one transferring hydrogen to the other:



In this reaction, alanine, aspartic acid, cysteine, glutamic acid, histidine, leucine, phenylalanine, serine, and valine act as hydrogen donors; arginine, glycine, hydroxyproline, ornithine, proline, and tryptophan act as hydrogen acceptors.

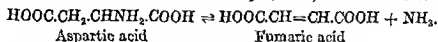
A somewhat similar oxido-reductive dismutation involving a single amino-acid is reported for *Clostridium propionicum* (Cardon, 1942; Cardon & Barker, 1947). The reaction for alanine is:



Similar reactions occur with serine and threonine. *Clostridium tetanomorphum* breaks down glutamic acid with the production of carbon dioxide, ammonia, hydrogen, acetic acid, and butyric acid (Woods & Clifton, 1937, 1938). The process is complex; its individual stages are not clearly understood.

### (iii) Deamination with desaturation

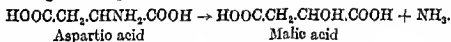
Aspartase, forming fumaric acid and ammonia from aspartic acid, occurs in bacteria (Quastel & Woolf, 1926; Virtanen & Tarnanen, 1932) and in higher plants (Virtanen & Tarnanen, 1932; Damodaran & Subramanian, 1948; Williams & McIntyre, 1955). The reaction is



The equilibrium is far to the side of fumaric acid.

### (iv) Hydrolytic deamination

Virtanen & Erkama (1938) found in *Bacterium fluorescens liquefaciens* both aspartase and another enzyme decomposing aspartic acid according to the equation:

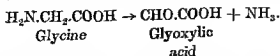


The reaction is stated to be catalysed by a single enzyme; malic acid could also arise from aspartic acid indirectly, e.g. via fumaric acid or oxalacetic acid. A somewhat similar transformation of tryptophan to indolelactic acid was reported by Ehrlich & Jacobsen (1911).

## F. The breakdown of Individual Amino-acids

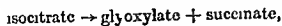
### (i) Glycine

A flavoprotein enzyme oxidizing glycine occurs in animal tissues (Ratner, Nocito, & Green, 1944) and in roots of *Vicia faba* (Robinson & Brown, 1952). The reaction is:

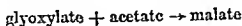


The enzyme appears to be specific for glycine, except that animal preparations attack sarcosine (methylglycine), forming glyoxylic acid and methylamine. The plant enzyme has not been tested on sarcosine. Glyoxylic acid is a metabolically important compound, taking part in

two key reactions of a sequence (glyoxylate cycle) which may be regarded as an extended tricarboxylic acid cycle and which provides a synthetic route from 2 carbon compounds to more complex substances. Glyoxylic acid is involved in the enzymatically catalysed steps

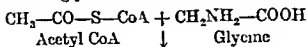


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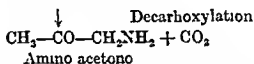


(Kornberg & Krehs, 1957, Wong & Aji, 1957)

Amino acetone is formed metabolically by *Staphylococcus aureus*, it could arise from glycine as follows (Elliott, 1959)



$\alpha$  Amino  $\beta$  ketobutyric acid



$\alpha$  Amino  $\beta$  ketobutyric acid may also arise by dehydrogenation of threonine. Amino acetone occurs, together with threonine, among the hydrolysis products of micrococcin P (Mijović & Walker, 1960)

#### (ii) Valine, isoleucine, leucine

The degradation of these branched chain amino acids has been thoroughly studied with animal tissues and enzyme preparations, little is known however, on the subject in plants. The available information will therefore be considered as briefly as its complexity permits. Some of the intermediates involved, e.g. tiglic acid and senecioic acid (dimethylacrylic acid) (Asahina, 1913), are known plant constituents. Several of the enzymes involved occur in micro-organisms such as *Aerobacter aerogenes*, *Neurospora crassa*, and *Tetrahymena pyriformis*.

Valine, on removal of its amino group by oxidative deamination or transamination, yields  $\alpha$  ketoisovaleric acid. This loses a molecule of carbon dioxide and is converted to the co-enzyme A derivative of isobutyric acid by a process similar to the formation of acetyl CoA from pyruvic acid. Several co-factors are probably involved, including lipoic acid. Isobutyryl CoA is dehydrogenated to methylacrylyl CoA,



which loses the elements of water to form  $\beta$ -hydroxyisobutyryl-CoA. Removal of co-enzyme A gives  $\beta$ -hydroxyisobutyric acid, which a DPN-dependent dehydrogenase oxidizes to methylmalonic semialdehyde. This compound probably forms methylmalonyl-CoA, which is decarboxylated to propionyl-CoA, a metabolic precursor of glucose in animal tissues (Kinnory, Takeda, & Greenberg, 1955; Robinson, Nagle, Bachhawat, Kupiecki, & Coon, 1957; Rendina & Coon, 1957). These reactions are summarized in Fig. 34.

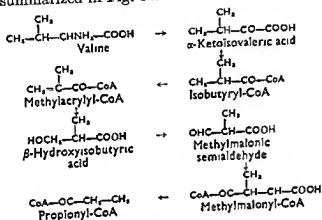


FIG. 34.

Isoleucine, on losing its amino group, gives  $\alpha$ -keto- $\beta$ -methylisovaleric acid. This forms  $\alpha$ -methylbutyryl-CoA by a process similar to that forming isobutyryl-CoA from the keto analogue of valine. The  $\alpha$ -methylbutyryl-CoA is dehydrogenated to tiglyl-CoA, which by loss of the elements of water leads to  $\alpha$ -methylacetoacetyl-CoA, which in turn yields acetyl-CoA plus propionyl-CoA (Coon & Abrahamsen, 1952; Coon, Abrahamsen, & Greene, 1954; Robinson, Bachhawat, & Coon, 1956). The reactions are summarized in Fig. 35.

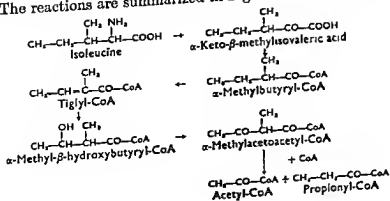


FIG. 35.

The keto analogue of leucine is  $\alpha$ -ketoisocaproic acid. This leads, by reactions analogous to those in the catabolism of valine and of isoleucine, to isovaleryl-CoA, dimethylacrylyl-CoA ( $\beta$ -methylcrotonyl-CoA,  $\beta$ -methylglutaryl-CoA), and  $\beta$ -hydroxyisovaleryl-CoA. The last-named compound is carboxylated by "active carbon dioxide" (possibly adenosine-5'-phosphoryl carbonate) to form  $\beta$ -hydroxy- $\beta$ -methylglutaryl-CoA, which is split to acetoacetic acid plus acetyl-CoA (Bachhawat, Robinson, & Coon, 1955, 1956; Bachhawat & Coon, 1957). The reactions are summarized in Fig. 36.

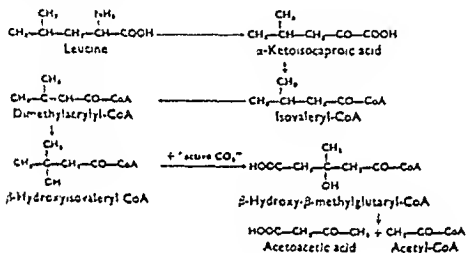
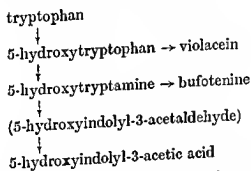


FIG. 36.

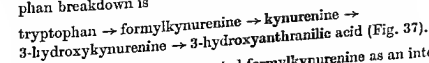
by an enzyme widespread in nature (Clark, Weissbach, & Udenfriend, 1954; Gaddum & Giarman, 1956; Buzard & Nytch, 1957). *Chromobacterium violaceum* forms 5-hydroxytryptophan from tryptophan (Mitoma, Weissbach & Udenfriend, 1955). The pigment from which the organism derives its specific name is a derivative of 5-hydroxyindole (Beer, Clarke, Khorana, & Robertson, 1948a; Beer, Jennings, & Robertson, 1954; Ballentyne, Barrett, Beer, Boggiano, Clarke, Eardley, Jennings, & Robertson, 1957) presumably formed from tryptophan via 5-hydroxytryptophan.

Udenfriend, Titus, Weissbach, & Peterson (1956) proposed the following scheme for the metabolism of tryptophan via 5-hydroxytryptophan:



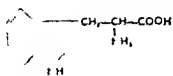
The last compound in this sequence (5-OH-IAA) is a normal constituent of the urino in toads and 7 species of mammals, including man (Erspamer, 1954, 1955). 5-Hydroxyindoleacetic acid and *N*-acetyl-5-hydroxytryptamine are also metabolites of 5-hydroxytryptamine in mammals (McIsaac & Page, 1959). 5-Hydroxyanthranilic acid, possibly related to these compounds, is a growth factor for some strains of *Escherichia coli* (Niemer & Oberdorfer, 1957). In contrast to the numerous derivatives of 5-hydroxytryptophan known as natural products, the only recorded derivatives of 4-hydroxytryptophan are psilocine and psilocybine, hallucinatory amines from the higher fungi *Psilocybe* and *Stropharia*. Psilocine is 4-hydroxydimethyltryptamine and psilocybine its phosphorylated derivative (Hofmann, Heim, Brack, & Kobel, 1958; Hofmann & Troxler, 1959).

In mammals and in *Neurospora crassa* a major pathway of tryptophan breakdown is

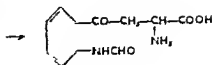


Knox & Mehler (1950) suggested formylkynurenine as an intermediate between tryptophan and kynurenine. This was confirmed (Makino &

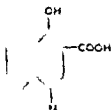
## BREAKDOWN OF AMINO ACIDS



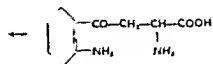
Tryptophan



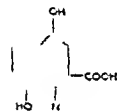
Formylkynurenine



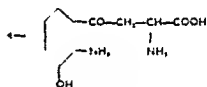
Kynurenine acid



Kynurenine



Xanthurenine acid



3-Hydroxykynurenine



3-Hydroxyanthranilic acid

in the urine of rabbits fed large amounts of tryptophan. Kynurenic acid, isolated from the urine of dogs by Liebig (1853) and shown by Ellinger (1904) to be a metabolic product of tryptophan in rats, is a side product of kynurenine. Its formation involves a transamination (Wiss, 1952; Miller, Tsuchida, & Adelberg, 1953) which is believed to produce 2-aminobenzoylpyruvic acid, its side-chain cyclizing to form kynurenic acid. Xanthurenic acid, isolated from urine of albino rats by Musajo (1935, 1937), is the 3-hydroxy derivative of kynurenic acid, and may arise from 3-hydroxykynurenine via 2-amino-3-hydroxybenzoylpyruvic acid. Xanthurenic acid is metabolized in the animal body, except in pyridoxine (vitamin B<sub>6</sub>) deficiency. It thus appears to be the starting point of an alternative route of tryptophan catabolism, the further course of which is not known. Other quinoline derivatives formed in mammals as metabolites of tryptophan include 6-hydroxykynurenic acid, quinaldic acid, and 8-hydroxyquinaldic acid (Roy & Price, 1959).

The first stage in tryptophan breakdown, its oxidation to formylkynurenine, involves both oxygen and hydrogen peroxide, as shown for rat liver (Knox & Mehler, 1950) and for bacteria (Hayaishi & Stanier, 1951). Formylkynurenine is hydrolysed to kynurenine by formylase, an enzyme found in liver (Knox & Mehler, 1950; Mehler & Knox, 1950) and in micro-organisms (Jakoby, 1954). Kynurenine is split to anthranilic acid and alanine by kynureninase, a pyridoxal phosphate-requiring enzyme (Kotake & Nakayama, 1941; Braunstein, Goryachenkova, & Pashkina, 1949; Dalglish, Knox, & Neuberger, 1951). The enzyme also splits alanine from formylkynurenine, 3-hydroxykynurenine, and 5-hydroxykynurenine, forming in each case the corresponding derivative of anthranilic acid.

Mitochondrial preparations from rat liver contain an enzyme, kynurenine hydroxylase, catalysing the formation of 3-hydroxykynurenine from kynurenine (Saito, Hayaishi, Rothberg, & Senoh, 1957); atmospheric oxygen is consumed in the reaction. 3-Hydroxykynurenine is an intermediate in the formation of eye-pigments, e.g. xanthommatine, in insects (Butenandt, Schiedt, Biekert, & Crommartie, 1954; Butenandt, Biekert, & Neubert, 1956). Xanthommatine contains the phenoxazone skeleton, otherwise known among natural products only in pigments from actinomycetes (Brockmann & Muxfeldt, 1955) and from the higher fungus *Trametes cinnabarinus* (Gripenberg, 1958). Kynurine (4-hydroxyquinoline), found in silkworm pupae, probably arises from kynurenine via kynuramine, whose side-chain cyclizes to

form the nitrogen containing ring of the quinoline (Butenandt, Karlson, & Zillig, 1951; Butenandt & Renner, 1953).

3 Hydroxyanthranilic acid appears to be a close precursor of nicotinic acid in animals, but the reactions involved in its formation are not entirely clear. Various workers have suggested that the ring of 3 hydroxyanthranilic acid is opened to form the unsaturated amino-acid aldhyde acroleinaminofumaric acid (Fig. 38), which is formed by

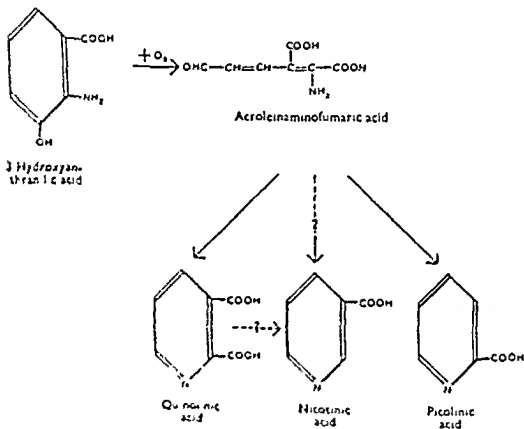


FIG. 38.

of the pyridine ring. Hankes & Segel (1958) found that the intact rat formed both quinolinic acid and *N*-methylnicotinamide from tritium-labelled tryptophan. Mobne, Walker, & Schweigert (1959) used an enzymatic preparation of rat liver on 3-hydroxyanthranilic acid labelled in the 3 position with  $C^{14}$ . They obtained quinolinic acid, labelled in the  $\alpha$ -carboxyl group only, which on non-enzymatic decarboxylation gave labelled carbon dioxide and inactive nicotinic acid.

In spite of these obscurities in detail, there is no doubt that in at least some mammals and fungi tryptophan is an important precursor of nicotinic acid. *Neurospora crassa* seems to form nicotinic acid exclusively from tryptophan (Partridge, Bonner, & Yanofsky, 1952). Some bacteria, however, lack kynureninase and do not form nicotinic acid from tryptophan, e.g. *Escherichia coli* and *Bacillus subtilis* (Yanofsky, 1954). How these species form nicotinic acid is not known. Its mode of formation in higher plants is also doubtful though tryptophan has been suggested as a precursor in excised leaves of broccoli, cabbage, and tomato (Gustafson, 1949), in sections of pea epicotyls (Galston, 1949a), and in germinating corn (*Zea mays*) (Nason, 1950). Kynurenine and 3-hydroxyanthranilic acid are also stated to be precursors of nicotinic acid in plants. Wiltshire (1953) found that slices from pea seedlings rapidly oxidized added tryptophan, and tentatively identified 3-hydroxykynurenine as a product.

The contention that in higher plants tryptophan is metabolized by a pathway leading to nicotinic acid is unconvincing; the reported data are inconclusive, and other evidence suggests that tryptophan is not a precursor. Bowden (1953) and Grimshaw & Marion (1958) found that in tobacco  $C^{14}$ -labelled tryptophan was not a precursor of the pyridine ring of nicotine, formed directly from nicotinic acid (Dawson, Christman, & D'Adamo, 1956; Dawson, Christman, D'Adamo, Solt, & Wolf, 1958). Henderson, Someroski, Rao, Wu, Griffith, & Byerrum (1959) found that  $C^{14}$ -labelled tryptophan was not a precursor of nicotinic acid in *Zea mays* or of nicotine in *Nicotiana rustica*. In higher plants, as in some bacteria, nicotinic acid may arise by some pathway other than that leading from tryptophan. This conclusion is supported by observations on the formation of trigonelline in the pea plant and the soybean. Nicotinic acid is an effective precursor (Zeijlemaker, 1953) of trigonelline, to which it is closely related. Trigonelline, however, is not formed from labelled tryptophan (Leete, Marion, & Spenser, 1955b) or labelled 3-hydroxyanthranilic acid (Aronoff, 1956a, b), which therefore seem not to be precursors of nicotinic acid.

In some bacteria (*Pseudomonas* spp.) (Hayaishi & Stanier, 1951) the breakdown of kynurenine occurs as follows:

kynurenine  $\rightarrow$  anthranilic acid  $\rightarrow$  catechol  $\rightarrow$

*cis*, *cis*-muconic acid  $\rightarrow$   $\beta$ -ketoadipic acid.

The  $\beta$ -ketoadipic acid is further metabolized by the enzymatic reactions (Katagiri & Hayaishi, 1957):

(1)  $\beta$ -ketoadipic acid + succinyl-CoA  $\rightleftharpoons$   $\beta$ -ketoadipyl-CoA + succinic acid,

(2)  $\beta$  ketoadipyl-CoA + CoA  $\rightleftharpoons$  succinyl-CoA + acetyl-CoA.

*Formation of indolyl-3-acetic acid and related compounds from tryptophan.* The formation from tryptophan of substances with auxin activity in higher plants has received much study. Some workers have tended to identify any compound with such activity as indolyl-3-acetic acid ( $\beta$ -indolylacetic acid, heteroauxin, IAA). This is confusing as other compounds, e.g. indolyl-3-acetonitrile (Jones, Henbest, Smith, & Bentley, 1952) and 5-hydroxytryptamine (Niaussat, Laborit, Dubois, & Niaussat, 1958) are active in auxin tests. Much recent work on the distribution and metabolism of IAA and its putative precursors and metabolites is based on chromatographic identifications, which are suggestive rather than final. These circumstances further complicate the involved problems in this field.

IAA (which figures in the older literature as skatole carboxylic acid) was recognized (Salkowski, 1884, 1885, 1899; Salkowski & Salkowski, 1880a, b) as a bacterial decomposition product of protein long before its importance as a hormone in higher plants was suspected. Hopkins & Cole (1903) showed that in pure cultures of *Escherichia coli* it arose, together with indole and indolyl-3-propionic acid (skatole-acetic acid), from tryptophan. It was detected in human urine by Herter (1908). Dunstan (1889) and Herter (1909) found the foul-smelling wood of *Celtis reticulosa* to contain indole and skatole; the latter author noted the possible presence of IAA.

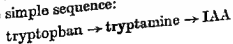
The growth-promoting properties for plant organs of IAA were first recognized with material extracted from human urine (Kögl, Haagen-Smit, & Erxleben, 1934) and from yeast (Kögl & Kostermans, 1934). Growth-promoting activity by indolyl-3-propionic acid was reported soon afterwards (Hitchcock, 1935). The amounts of IAA in tissues of higher plants are very small, Haagen-Smit, Dandliker, Wittwer, & Murneck (1940) isolated 101 mg from 100 kg of immature kernels of corn (*Zea mays*). Subsequent work, using mainly chromato-



graphic methods, demonstrated it in many but not all of the species examined. Plant organs in which IAA has been sought but not detected include coleoptiles of barley, maize, and oats; hypocotyls of buckwheat, cucumber, pea, and sunflower; stems of cabbage, pea, and tomato; and potato sprouts (Good, Andreae, & Van Ysselstein, 1956). It is also reported absent from tissue cultures derived from tubers of *Helianthus tuberosus* (Jerusalem artichoke) (Schoen & Morel, 1954), though these form other auxins of unknown constitution which apparently lack the indole nucleus.

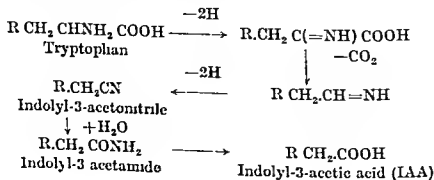
There is an impressive body of evidence that in fungi (Thimann, 1935) and a considerable range of higher plants (Skoog, 1937; Wildman, Ferri, & Bonner, 1947; Kulescha, 1949; Henderson & Bonner, 1952) tryptophan is converted to active growth substances. The mechanism of this conversion remains uncertain. Went & Thimann (1937) suggested indolyl-3-acetaldehyde as a possible intermediate, an idea supported by several subsequent workers who showed that besides the acidic IAA a neutral auxin occurred in plant tissues. This substance was often equated with indolyl-3-acetaldehyde, but the isolation of another neutral auxin, indolyl-3-acetonitrile (Jones, Henbest, Smith, & Bentley, 1952), made it clear that the identification was not necessarily correct. Critical chromatographic studies (Linser, Mayr, & Maschek, 1953), and finally isolation from aqueous extracts of cabbage (Jones & Taylor, 1957) have, however, shown that both the aldehyde and the nitrile occur in plants. The nitrile has been detected chromatographically in various plants (Fischer & Behrens, 1953; Bennet-Clark & Kefford, 1953).

Other compounds reported in plants and related to tryptophan and the auxins include indolyl-3-carboxylic acid (Jones & Taylor, 1957), indolyl-3-propionic acid (Linser, Mayr, & Maschek, 1953), indolyl-3-pyruvic acid (Stowe & Thimann, 1953), and indolyl-3-butyric acid (Blommaert, 1954). The crown gall organism (*Agrobacterium tumefaciens*) forms indolyl-3-pyruvic acid, indolyl-3-lactic acid, and tryptophol from tryptophan (Kaper & Velstra, 1958). Their metabolic relationships are largely unknown. Indolyl-3-acetaldehyde is readily converted to IAA in oat coleoptiles (Larsen, 1949; Bentley & Housley, 1952). Intact animals and surviving animal organs form IAA from tryptamine (Ewins & Laidlaw, 1913; Guggenheim & Loeffler, 1916). An amine oxidase from pea seedlings also oxidizes tryptamine to IAA (Clarke & Mann, 1957). The simple sequence:



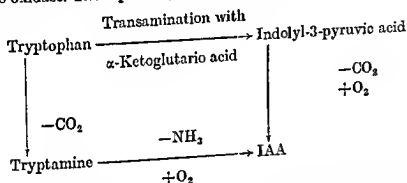
thus seems possible in plants. The enzymatic conversion of tryptamine to an active auxin, presumably IAA, has indeed been demonstrated for pineapple leaves (*Ananas*) (Gordon & Nieva, 1949) and for bean plants (*Phaseolus*) (Weintraub, Brown, Nickerson, & Taylor, 1952). Tryptamine, however, appears not to be a common plant constituent, though recorded from three species of *Acacia* (White, 1944). An amine oxidase probably identical with that converting tryptamine to IAA occurs in many plants, particularly legumes, but is absent from others (Werle & Zabel, 1948), including all gymnosperms and monocotyledons tested. Enzymes decarboxylating tryptophan to tryptamine seem to be rare in organisms generally, not only in higher plants. They have, however, been detected in bacteria (Berthelot & Bertrand, 1912b; Weissbach, King, Sjoerdsma, & Udenfriend, 1959) and in animal tissues (Weissbach *et al.*, 1959), and may be more widely distributed than is now recognized. Pyridoxal enzymes decarboxylating 5-hydroxytryptophan to 5-hydroxytryptamine are also known from bacteria and from animal tissues (Clark, Weissbach, & Udenfriend, 1954; Gaddum & Giarman, 1956; Udenfriend, Titus, Weissbach, & Peterson, 1956; Buzard & Nytech, 1957). These authors cite some evidence for the occurrence of 5-hydroxyindoleacetic acid in plants; it is a normal constituent of human urine (Erspamer, 1955; Udenfriend, Titus, & Weissbach, 1955). Plants may contain a set of hydroxyindole compounds corresponding to the known indole derivatives; sporadic occurrences of 5-hydroxytryptamine and some of its derivatives are mentioned in the chapter on alkaloids. Little is known of their physiology in the plant; 5-hydroxytryptamine (serotonin) is an important animal hormone (Woolley, 1957).

As tryptamine seems unlikely to be a generally occurring intermediate in the formation of IAA in plants, some other pathway must be sought. The following sequence (Jones *et al.*, 1952) has been suggested, but still lacks experimental verification:

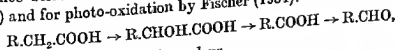


Kutáček, Procházka, & Grünberger (1960) showed intact cabbage plants to form indolyl-3-acetonitrile, indolyl-3-carboxylic acid, indolyl-3-pyruvic acid, and ascorbigen (an indolyl derivative of ascorbic acid) from labelled tryptophan.

For animal tissues and intestinal bacteria Weissbach, King, Sjoerdsma, & Udenfriend (1959) demonstrated two routes for the formation of IAA from tryptophan. Quantitatively the more important route is by transamination of tryptophan with  $\alpha$ -ketoglutaric acid, forming indolepyruvic acid, which on decarboxylation and oxidation yields IAA. An alternative pathway is via the decarboxylation of tryptophan to tryptamine, followed by its conversion to IAA by monoamino oxidase. These pathways are shown below:

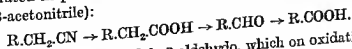


The breakdown of IAA both by photo-oxidation (Galston, 1949b; Brauner, 1953; Goldacre, 1954) and enzymatically (Larsen, 1936) has been extensively studied. Ultraviolet irradiation causes oxidation of IAA; its oxidation by visible light is accelerated by riboflavin. The sequence below was suggested for enzymatic oxidation by Goldacre (1951) and for photo-oxidation by Fischer (1954):



where R represents the indole nucleus.

Later work (Fawcett, Taylor, Wain, & Wightman, 1958) has demonstrated in pea and wheat tissues the sequence (beginning with indolyl-3-acetonitrile):



IAA is decarboxylated to indole-3-aldehyde, which on oxidation forms indole-3-carboxylic acid. The enzyme hydrolysing the nitrile to IAA is absent in tubers of *Helianthus tuberosus* (Nitsch & Nitsch, 1959).

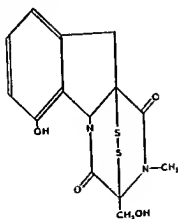
Neuberg (1908) exposed tryptophan solutions to sunlight and noted the formation of a volatile substance "possibly indolyl-3-acetaldehyde".

Berthelot & Amoureux (1938) showed that ultra-violet irradiation of tryptophan led to the formation of IAA. This was confirmed by Melchior (1957), who found that photolysis of tryptophan by visible light and ultra-violet rays formed tryptamine, tryptophol, indolyl-3-acetic acid, indole-3-aldehyde, indole-3-carboxylic acid, indole, skatole, anthranilic acid, and unidentified substances containing the indole group. Kynurenine and 3-hydroxykynurenine are breakdown products of irradiated tryptophan (Yoshida & Kato, 1954). Hakim & Thiele (1960) identified formylkynurenine as an intermediate in the formation of kynurenine from tryptophan by ultra-violet radiation. The photolytic breakdown of tryptophan is obviously complex; its stages do not necessarily correspond to those occurring in the plant.

The fungus *Omphalia flavid*a contains an enzyme oxidizing IAA (Sequeira & Steeves, 1954; Ray & Thimann, 1956). *O. flavid*a is a destructive parasite of coffee in tropical America, causing extensive defoliation attributed to its interference with auxin metabolism in the leaves. The IAA-oxidizing enzyme is also a peroxidase, catalysing the oxidation of phenols with hydrogen peroxide as electron acceptor. Various monophenols stimulate the oxygen-consuming oxidation of IAA by the enzymes of pea homogenates (Goldacre, Galston, & Weintraub, 1953) and by purified peroxidase from horseradish (*Cochlearia armoracia*) (Kenten, 1955). IAA-oxidizing systems with peroxidase activity also occur in bean (*Phaseolus vulgaris*) roots (Kenten, 1955) and in seedlings of *Lupinus albus* (Stutz, 1957).

Many alkaloids structurally related to indole may be metabolically derived from tryptophan. Indole itself seems rarely to accumulate in plant tissues, but is recorded from oils of jasmine (Hesse, 1904) and of orange flowers (Hesse & Zeitschel, 1902). Its presence in fresh orange flowers was confirmed by Stowe, Thimann, & Kefford (1956). Skatole (3-methylindole), known as a product of protein breakdown by bacteria, is reported from cabbage (Linser, Mayr, & Maschek, 1953). Biosynthesis of the ergot alkaloids, which are rather complex derivatives of indole, is discussed in Chapter 12. Another fungal product related to indole, ghotoxin (Fig. 39) from *Trichoderma viride*, arises from phenylalanine (Suhadolnik & Chenoweth, 1958).

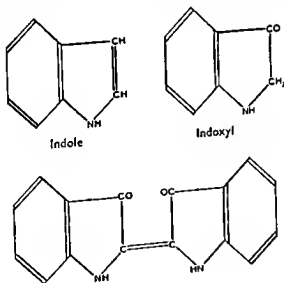
Indigo, another indole derivative, played an important rôle in the development of organic chemistry owing to its study by early workers, e.g. Chevreul (1808a, b, 1809). Indigo is a blue dye known since antiquity as a product in Europe of woad (*Isatis tinctoria*, Cruciferae) and in Asia of *Indigofera tinctoria* (Leguminosae) and other species of the same



Gliotoxin

FIG. 39.

genus; it is known also from *Polygonum tinctorium* (Polygonaceae) and from some orchids. The dye as such does not exist in the plants; the glucosido indican breaks down enzymatically in macerated tissues yielding glucose and indoxyl, which in the presence of atmospheric

Indigo  
FIG. 40.

oxygen oxidizes spontaneously to indigo (Fig. 40). The pigment Tyrian purple from the molluscs *Murex* and *Purpura* is a dibromoindigo (Friedländer, 1909, 1922); it probably arises from a bromoindoxyl (Bouchilloux & Roche, 1955).

(iv) *Histidine*

Urocanic acid (imidazole-4-acrylic acid), obtained by Jaffe (1874) and Siegfried (1898) from dog urine and considered an abnormal metabolite, is now recognized as a regular intermediate in the breakdown of histidine by bacteria (Raistrick, 1917; Darby & Lewis, 1942) and by mammals (Hunter, 1912; Konishi, 1922; Kiyokawa, 1933). Hunter (1912) showed that urocanic acid was identical with a compound which Barger & Ewins (1911) obtained from ergothioneine and named  $\beta$ -2-glyoxaline-4-acrylic acid; its close structural relation to histidine was thus established.

Cell-free extracts of *Pseudomonas fluorescens* convert histidine to glutamic acid and formic acid with the production of two molecules of ammonia per molecule of histidine (Tabor & Hayaishi, 1952). The occurrence of urocanic acid as an intermediate in this process was demonstrated using histidine labelled with  $C^{14}$  and with  $N^{15}$  (Tabor, Mehler, Hayaishi, & White, 1952). It was also found that extracts

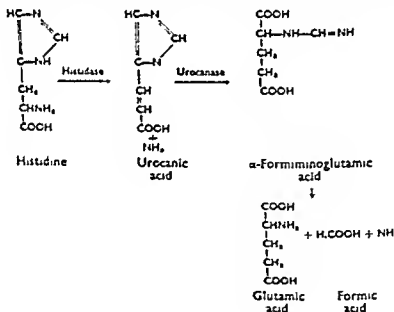
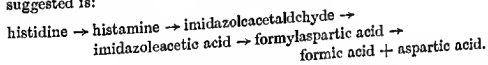


FIG. 41.

subjected to rather severe heat treatment (15 minutes at  $85^\circ\text{C}$ ) catalysed the formation of urocanic acid without further breakdown. There is evidence (Walker & Schmidt, 1944; Borek & Waelsch, 1953) that formiminoglutamic acid is an intermediate in the breakdown of urocanic acid. This pathway of histidine breakdown (Fig. 41) has been studied in *Leuconidium tetanomorphum* (Wachsmann & Barker, 1955) and

*Aerobacter aerogenes* (Magasanik & Bowser, 1955) as well as in *Pseudomonas fluorescens*. Miller & Waelsch (1957*a, b*) suggested 5-imidazolone-4-acrylic acid and 5-imidazolone-4-propionic acid as intermediates between urocanic acid and formiminoglutamic acid in cat liver. Breakdown of formiminoglutamic acid to glutamic and formic acids in mammals involves folic acid derivatives. The reaction in cat liver has been clarified by Miller & Waelsch (1957*c, d*). Formiminoglutamic acid is excreted (Broquist, 1956) in the urine of human patients treated for leukaemia with folic acid antagonists.

Other pathways of histidine breakdown are also known. Roche, Thoai, & Glahn (1954) found that the hepatopancreas of the mussel *Mytilus edulis* converted histidine to several substances retaining the imidazole ring. These include imidazolepyruvic acid ( $R-CH_2-CO-COOH$ ), imidazoleacetic acid ( $R-CH_2-COOH$ ), imidazoleacetaldehyde ( $R-CHO$ ), imidazolemethanol ( $R-CH_2OH$ ), and imidazolecarboxylic acid ( $R-COOH$ ). The symbol R in these abbreviated formulae represents the imidazolyl group; histidine, on this convention, is  $R-CH_2-CHNH_2-COOH$ . Imidazoleacetic acid also figures in the breakdown of histidine by *Pseudomonas* (Hayaishi, Tabor, & Hayaishi, 1954; Tabor & Hayaishi, 1955). The pathway suggested is:



Kapeller-Adler & Fletcher (1959) showed that an enzyme from pig kidney oxidized histamine to imidazoleacetaldehyde, further oxidized to imidazoleacetic acid. The aldehyde formed in the enzymatic reaction was identified by comparison with the synthetic compound prepared by oxidation of histamine with sodium hypochlorite (Langheld, 1909). In *Escherichia coli* aminoimidazolecarboxamide, a precursor of purines, is probably derived from histidine (Hedegaard, Beau-Thomé, Thoai, & Roche, 1959). Imidazoleacetic acid, imidazolelactic acid, imidazolepropionic acid, and urocanic acid occur in the slug *Arion empiricorum* (Ackermann & Menssen, 1960b); 1,3-dimethylimidazoleacetic acid betaine (zooanemonine) from sea anemones probably also arises in histidine catabolism (Ackermann & List, 1960).

Little is known of histidine catabolism in higher plants. Some contain bistamine, possibly arising by decarboxylation of histidine; it is oxidized by diamino oxidase to imidazoleacetaldehyde, which could

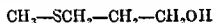
be further metabolized as in *Pseudomonas* via imidazoleacetic acid, traces of which are recorded in *Spinacia oleracea* (Appel & Werle, 1959). This pathway is unlikely to be general in higher plants. Some lack diamine oxidase, and only a few are known to contain histamine. There is at present no evidence regarding alternative pathways of histidine breakdown in higher plants.

(v) *Methionine and cysteine*

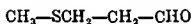
The metabolism of these amino-acids also is known mainly from studies on micro-organisms and on mammalian tissues. The first step in the breakdown of methionine is formation of the corresponding keto-acid ( $\alpha$ -keto- $\gamma$ -methylthiolbutyric acid) by transamination (Cammarata & Cohen, 1950; Wilson, King, & Burris, 1954) or by amino-acid oxidase (Blanchard, Green, Nocito, & Ratner, 1945). This keto-acid is:



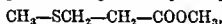
The related alcohol:



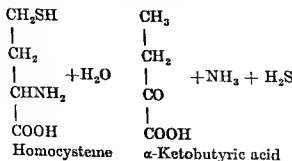
and aldehyde:



occur in shoyu (Japanese soy sauce) (Akabori & Kaneko, 1936); the aldehyde is reported also in milk exposed to light (Anonymous, 1953). Another related compound, methyl 3-methylthiolpropionate:

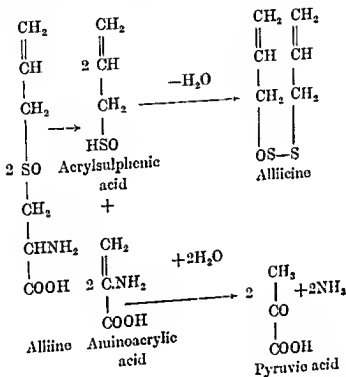


occurs in pineapple (*Ananas comosus*) (Haagen-Smit, Kirchner, Deasy, & Prater, 1945). The keto-acid is broken down in animal tissues to methyl mercaptan ( $\text{CH}_3\text{SH}$ ) and homoserine. Methionine is demethylated to homocysteine, which may be oxidized to homocystine and homocystic acid (Medes & Floyd, 1942). It is also broken down by bacterial and mammalian enzymes to  $\alpha$ -ketobutyric acid, with the formation of ammonia and hydrogen sulphide (Fromageot & Desnuelle, 1942; Kallio, 1951):





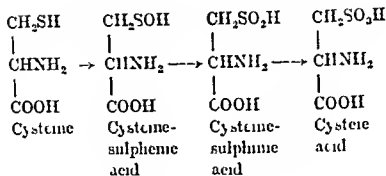
In mammals  $\alpha$ -ketobutyric acid may be aminated to form  $\alpha$ -aminobutyric acid (Matsuo & Greenberg, 1953). The corresponding reactions with cysteine (Tarr, 1933; Fromageot, Wookey, & Chaix, 1940) form pyruvic acid and alanine. The breakdown of alliin in macerated onion bulbs, as formulated by Stoll & Seebeck (1949), is somewhat similar:



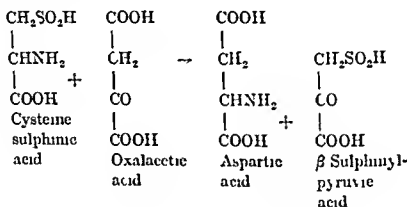
Alliine is a bactericidal non-odorous substance which gives rise to allyl sulphides with the characteristic odour of onion (Cavallito, Buck, & Suter, 1944; Stoll & Seebeck, 1947, 1949). It inhibits numerous enzymes (mostly with sulphydryl groups) at a concentration of 0.0005 M (Wills, 1956). Alliine is broken down by a specific enzyme, alliinase; its prosthetic group is pyridoxal phosphate (Goryachenkova, 1952).

Cysteine is oxidized enzymatically to cystine by cytochrome oxidase (Keilin, 1930) and by an enzyme dependent on diphosphopyridine nucleotide (Romane & Nickerson, 1954). It is also oxidized to cysteinesulphinic acid (Pirie, 1934; Medes & Floyd, 1942), formed by the intact rat from cysteine labelled with  $\text{S}^{32}$  (Chapeville & Fromageot, 1955). Cysteinesulphinic acid is probably an unstable intermediate between cysteine and cysteinesulphonic acid; the latter can be further oxidized to cysteic acid:

## BREAKDOWN OF AMINO-ACIDS

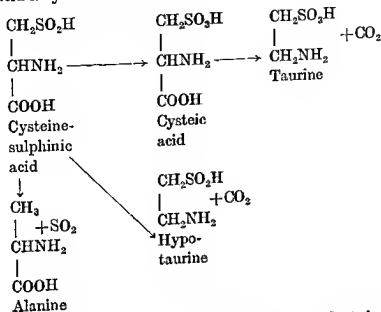


Cystein sulphinic acid appears to be a normal metabolite in the rat (Bergeret & Chatagner, 1954). It transaminates, in preparations from various animal organs, with oxalacetic acid and  $\alpha$  ketoglutaric acid,  $\beta$  sulphinylpyruvic acid is formed, together with aspartic acid or glutamic acid (Kaerney & Singer, 1953, Chatagner, Bergeret, S  journe, & Fromageot, 1952).



$\beta$  Sulphinylpyruvic acid has not been isolated it is believed to break down spontaneously to pyruvic acid and sulphite, which is oxidized to sulphate. Loss of sulphite from cysteine sulphinic acid resembles decarboxylation in being reversible (Chapeville & Fromageot, 1954), the reverse reaction may incorporate inorganic sulphur into organic compounds. Cystein sulphinic acid is also broken down by enzymes from liver to alanine and sulphur dioxide (Fromageot & Grand, 1943, Fromageot, Chatagner, & Bergeret, 1948, Bergeret & Chatagner, 1952). The reactions splitting off carbon dioxide and sulphur dioxide both occur in intact animals (Bergeret Chatagner, & Fromageot, 1952). Extracts of oat leaves catalyse the breakdown of cystein sulphinic acid. It transaminates with  $\alpha$  ketoglutaric acid, giving  $\beta$  sulphinyl pyruvic acid, which eliminates sulphite to form pyruvic acid (Perez-

Milan, Sehliack, & Fromageot, 1959). The catabolism of cysteinesulphinic acid may be summarized:

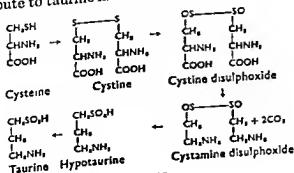


Alanine

Taurine is usually considered a metabolic end-product, but in the rat it is metabolized to carbamyltaurine and guanidotaurine (Theoi, Roche, & Olomucki, 1954).

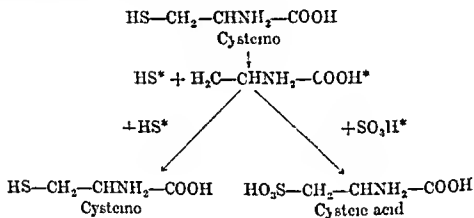
Cysteic acid, the most oxidized product of cysteine breakdown, is decarboxylated (Blaschko, 1942) by animal enzymes to taurine, which is widely distributed in animals and reported also from some algae. Cysteinesulphinic acid is similarly decarboxylated to hypotaurine; both enzymes require pyridoxal phosphate (Bergeret & Chatagner, 1952; Hope, 1955).

Cysteinesulphinic acid can be oxidized to cysteic acid, and hypotaurine to taurine, the  $\text{—SO}_2\text{H}$  group of each being converted to  $\text{—SO}_3\text{H}$ . Another route to taurine in animal tissues (Pirie, 1934; Medes, 1939)



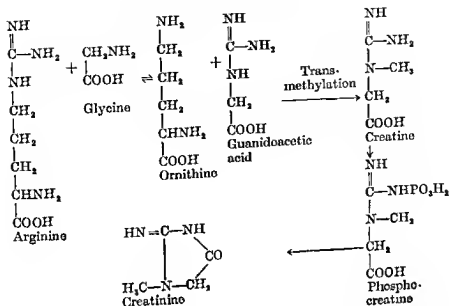
**FIG. 42.**

is cysteine  $\rightarrow$  cystine  $\rightarrow$  cystine disulphoxido  $\rightarrow$  cystamino disulph oxido  $\rightarrow$  hypotaurino  $\rightarrow$  taurine (Fig 42) Cystamine disulphoxido is formed from cysteine in rats (Cavallini, Mondovì, & Do Marco, 1952) possibly by decarboxylation of cystine disulphoxide which is readily metabolized in animals (Medes, 1937) Embryonated hen eggs synthesize taurine from sulphate sulphur (Miehlin, Pearson & Denton 1955) The sulphate is first reduced to sulphite, this combines with an aminated 3 carbon compound to form cysteic acid, which is decarboxy lated to taurine (Chapeville & Fromageot, 1957) The primary reaction appears to involve the splitting of cysteine to a sulphydryl radical and another free radical which reacts either with sulphydryl to regenerate cysteine or with a sulphite radical to form cysteic acid, as in the scheme below (Chapeville & Fromageot, 1958)



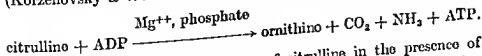
#### (vi) Arginine

The breakdown of arginine to ornithine and urea by arginase is a stage in the urea cycle of Krebs and Henseleit, a major pathway of urea formation in animals and probably in plants. Crystalline arginase has been prepared from beef liver (Bach & Killip 1958). In animal tissues arginine also yields ornithine by a transamidation reaction with glycine the other product being guanidoacetic acid (glycocyamine) (Borsook & Dubnoff 1941, Bloch & Schoenheimer 1941). Guanidoacetic acid forms creatine by transmethylation in the liver (Borsook & Dubnoff 1940). A similar transmethylation of added guanidoacetic acid is reported in etiolated wheat seedlings the methyl groups being supplied by methionine (Barrenscheen & Pany 1942, Barrenscheen & von Valy, Nagi 1942). In animal tissues creatine probably leads to creatinine via phosphocreatine (Borsook & Dubnoff, 1947a b).



Guanidoacetic acid seems unknown as a plant constituent; creatine is recorded from cocoa (*Theobroma cacao*) (Mitchell, Beadles, & Keith, 1926).

Several other pathways of arginine breakdown are known in micro-organisms and in animals. Many bacteria decompose arginine to carbon dioxide and ammonia without forming urea (Hills, 1949; Oginsky & Gehrig, 1952; Schmidt, Logan, & Tytell, 1952). The first step, as in yeast (Roche & Lacombe, 1952), is an enzymatic hydrolysis of arginine to citrulline and ammonia. The citrulline is hydrolysed to ornithine, carbon dioxide, and ammonia by another enzyme requiring inorganic phosphate, magnesium ions, and either adenosine diphosphate or adenylic acid; adenosine triphosphate is formed during the hydrolysis (Korzenovsky & Werkman, 1953; Knivett, 1954):

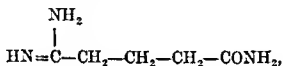


A somewhat similar breakdown of citrulline in the presence of phosphate or arsenate occurs in preparations of mammalian liver (Krebs, Eggleston, & Knivett, 1955). The corresponding hydrolysis of canavanine to *O*-ureidohomoserine in extracts of *Streptococcus faecalis* appears to be catalysed by the same enzyme as attacks arginine (Kihara & Snell, 1957).

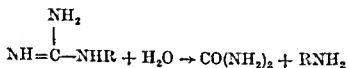
Enzymes deaminating arginine to the corresponding keto acid,  $\alpha$ -keto- $\delta$ -guanidovaleric acid, occur in tissues of birds (Boulanger &

Osteux, 1955, 1956) and insects (Garcia, Roche, & Tixier, 1956, Garcia, Couerbe, & Roche, 1957) The deamination is catalysed by an L amino acid oxidase, the keto acid being further transformed to  $\gamma$  guanidobutyric acid by hydrogen peroxide in the tissues Various invertebrates form  $\gamma$  guanidobutyric acid (Thoai, Roche, & Robin, 1952, Robin & Thoai, 1957), other animal products apparently related to the catabolism of arginine include  $\delta$  guanidovaleric acid (Thoai & Lacombe, 1958) and  $\gamma$  guanidobutyramide (Thoai, Robin, & Pradel, 1957)

In *Streptomyces griseus* (Thoai, Hatt, & An, 1955, 1956, Roche, Thoai, & Hatt, 1956, Thoai, Hatt, An, & Roche, 1956)  $\gamma$  guanidobutyramide,



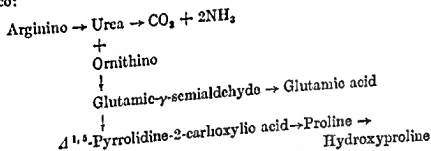
is formed from arginine by oxidative carboxylation and hydrolysed (Thoai & An, 1956) to  $\gamma$  guanidobutyric acid by a specific enzyme, guanidobutyramidase *S. griseus* also enzymatically hydrolyses a wide variety of monosubstituted guanidines (arginine, guanidoacetic acid, guanidopropionic acid, guanidobutyric acid, streptidine, and streptomycin) The enzyme differs from arginase in its low specificity (if a single enzyme is really involved) and in its optimum pH The general reaction is



Removal in this way of the guanido group of  $\gamma$  guanidobutyric acid (Kobayashi, 1947) leads to  $\gamma$  aminobutyric acid, formed also by decarboxylation of glutamic acid

The breakdown of arginine has been less studied in higher plants than in other organisms Kiesel (1909) showed that arginine disappeared during autolysis of seedlings of *Lupinus luteus*, probably breaking down to guanino by an oxidative process Guanino was found earlier in etiolated seedlings of *Vicia faba* by Schulze (1893) who regarded it as formed by the oxidation of protein presumably via arginine produced on hydrolysis Klein & Lauböck (1932a, b) found an increase of free arginine during germination and seedling development in several species, including *Canavalia ensiformis*, *Cucumis sativus*, *Lupinus albus*, *Phaseolus vulgaris*, *Pinus pinea* and *Pisum sativum* This increase,

however, was less than the amount of arginine formed by protein hydrolysis. Some arginine arising by hydrolysis must therefore have been metabolized further. Klein & Tauböck (1932b) showed that in sterile culture seedlings of *Zea* and *Phaseolus* absorbed arginine unchanged through the roots, and metabolized it with the formation of urea. Duranton (1958) studied the breakdown of  $C^{14}$ -labelled arginine in auxin-stimulated tissue cultures of vascular parenchyma from Jerusalem artichoke (*Helianthus tuberosus*). After 48 hours, radioactive carbon from uniformly labelled arginine appeared in proline (45 per cent), hydroxyproline (20 per cent), and glutamic acid (5 per cent). Alanine, aspartic acid, glutamic acid, asparagine, and glutamine also received some carbon from the arginine. When the arginine supplied was labelled only in the amidino carbon atom, all the radioactivity appeared in carbon dioxide. The author suggested the following sequence:



Tissue cultures of carrot root stimulated with coconut milk formed proline and hydroxyproline which were rapidly incorporated into a metabolically inactive protein (Steward, Pollard, Patchett, & Witkop, 1958).

The pathway of arginine breakdown in tumorous tissues of *Helianthus tuberosus* is different from that in cultures of normal tissues. Tumorous tissues, in contrast to normal, grow *in vitro* without added auxin. They produce (Morel & Duranton, 1958) from arginine large amounts of lysopine, an amino-acid first discovered by Lioret (1957a, b) in tissue cultures of *Scorzonera*; six compounds reacting with the Sakaguchi reagent, and therefore presumably monosubstituted guanidines, were detected as metabolites of arginine. None of these was found in normal tissues. The most abundant was identified as  $\gamma$ -guanidobutyric acid (Morel & Duranton, 1958);  $\alpha$ -hydroxy- $\delta$ -guanidovaleric acid was also present. Little proline was found and no hydroxyproline.

(vii) *Lysine and ornithine*

Preparations from mammalian liver convert lysine to  $\alpha$  aminoadipic acid,  $\alpha$  keto adipic acid, and glutaric acid, probably in that order (Borsook Deasy, Haagen Smit, Keighley, & Lowy, 1948) Cyclic compounds are also prominent metabolites of lysine  $C^{14}$  labelled lysine is converted to pipercolic acid in *Phaseolus vulgaris* (Lowy, 1953,

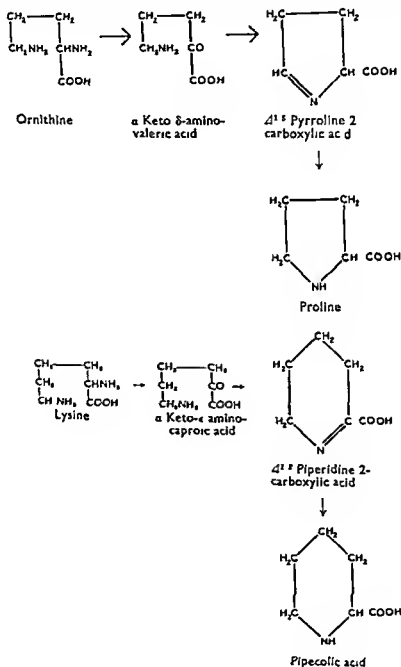


FIG. 43



Grobbelaar & Steward, 1953), *Neurospora crassa* (Schweet, Holden, & Lowy, 1954), the rat (Rothstein & Miller, 1954) and the turkey (Boulanger & Osteux, 1952, 1955, 1956; Boulanger, Coursaget, Bertrand, & Osteux, 1957). Turkey liver contains an amino-acid dehydrogenase fairly specific for the basic amino-acids arginine, ornithine, and lysine. It forms  $\alpha$ -keto- $\delta$ -guanidovaleric acid from arginine and  $\alpha$ -keto- $\delta$ -aminovaleric acid from ornithine.  $\alpha$ -Keto- $\delta$ -aminovaleric acid is in equilibrium with its cyclic form,  $\Delta^{1,5}$ -pyrrolino-2-carboxylic acid, which on reduction yields proline.  $\alpha$ -Keto- $\epsilon$ -aminocaproic acid, formed from lysine by amino-acid dehydrogenase, exists largely in the cyclic form as  $\Delta^{1,2}$ -piperidino-2-carboxylic acid and yields pipercolic acid on reduction (Fig. 43). The enzyme from turkey liver also decaminated 5-hydroxylysine to a product giving 5-hydroxypipercolic acid on reduction (Boulanger, Osteux, & Bertrand, 1958).

#### (viii) Proline and hydroxyproline

Animal tissues convert proline to glutamic acid (Weil-Malherbe & Krebs, 1935; Neber, 1936). Experiments with enzyme systems of animal origin (Taggart & Krakaur, 1949; Lang & Schmid, 1951; Smith & Greenberg, 1957) indicated that proline was dehydrogenated to a pyrrolinecarboxylic acid, a cyclic compound in equilibrium with its open-chain analogue, glutamic semialdehyde, which is readily oxidized to glutamic acid. Adams, Friedman, & Goldstone (1958) found that liver preparations converted hydroxyproline to  $\gamma$ -hydroxyglutamic semialdehyde and  $\gamma$ -hydroxyglutamic acid. Adams (1959) isolated from soil a strain of *Pseudomonas striata* metabolizing hydroxyproline to  $\alpha$ -ketoglutaric acid. An initial enzymatic epimerization of L-hydroxyproline to D-allohydroxyproline was followed by oxidation of the latter compound to  $\alpha$ -keto- $\gamma$ -hydroxy- $\delta$ -aminovaleric acid, which was further metabolized to  $\alpha$ -ketoglutaric acid and glutamic acid. Pyrrole-2-carboxylic acid, formed by an irreversible side reaction, was not utilized either in extracts or in intact cells. Brewers' yeast and wheat germ extracts appeared unable to metabolize hydroxyproline.

## CHAPTER 10

# AMIDES AND OTHER SOLUBLE NITROGEN-STORING SUBSTANCES

### A. AMIDES

#### A. General

Ammonia holds a key place in nitrogen metabolism. The free base is, however, toxic except in very low concentrations (Cloëz & Gratiolet, 1851; Takabayashi, 1897-8; Naftel, 1931) and does not accumulate in the cell. Compounds storing ammonia in a harmless form and releasing it when required are thus important metabolites. Many workers have ascribed this function essentially to asparagine, replaced in some species by glutamine, though their functions are not completely interchangeable. Compounds which may replace or supplement the amides as reserves of readily available nitrogen include urea and its metabolically related amino-acids (arginine, citrulline, *N*-acetylornithine) and ureides (allantoin, allantoic acid); in some species such compounds as azetidine-2-carboxylic acid and  $\gamma$ -methylcne glutamic acid may be reserve materials.

#### B. The Amino-acid Amides Asparagine and Glutamine in Seedlings

Asparagino crystallizes from plant juices as the characteristic monohydrate, isolated under various names by Delaville (1802), Vauquelin & Robiquet (1806), and other early workers. Plisson (1827) correlated these observations and converted asparagino to aspartic acid, whose structure was established (Kolbe, 1862) after its synthesis by dehydration of ammonium malate (Dessaigues, 1850a; Wolff, 1850; Pasteur, 1852). Piutti (1888a) identified asparagino as the  $\beta$ -amide of aspartic acid; it may exist as more than one isomer. Ritthausen (1869) obtained aspartic acid, and also the previously unknown glutamic acid, by acid hydrolysis of pea seed proteins. Von Knierem (1875) prepared aspartic acid by enzymatic hydrolysis of gluten. Glutamino was first isolated from beetroot (Schulze & Urich, 1877) and from pumpkin seedlings (Schulze & Barbicri, 1877); beetroot is still a favourite source. Piria (1844, 1848) showed that asparagino accumulated in vetch

(*Vicia sativa*) seedlings both in the light and the dark, and suggested that it arose from protein. It disappeared from plants in the light when they reached the flowering stage, as confirmed by Pasteur (1851). Sullivan (1858) showed that asparagine slowly disappeared in etiolated seedlings transferred to the light. Dessaignes & Chautard (1848) confirmed Piria's observations on asparagine in the vetch, and extended them to other species.

Piria (1844) obtained ammonium succinate by bacterial putrefaction of asparagine. Its metabolic connexion with the 4-carbon-atom dicarboxylic acids was thus suspected even before its chemical relation to malic acid was established. Boussingault (1864, 1868) made extensive quantitative studies on seedlings germinating without an external supply of nitrogen. Seedlings grown in the light contained more carbon, hydrogen, and oxygen than the original seeds; those in the dark lost each element. Nitrogen was unchanged in both groups. Boussingault noted the analogy, much stressed by later workers, between urea in animals and asparagine in plants. Animals excrete as urea part of the nitrogen ingested in protein; plants excrete very little nitrogen, but may accumulate asparagine as a reserve of nitrogen for later use. Boussingault associated the disappearance of asparagine with photosynthesis, a view confirmed by Pfeffer (1873), who showed that accumulated asparagine remained unchanged in plants kept in the light in an atmosphere free from carbon dioxide.

Beyer (1867) found that almost all the nitrogen in seeds of *Lupinus luteus* was in protein, which decreased during germination with a concurrent increase in asparagine. He suggested that this arose partly from protein and partly by combination of ammonia with malic acid, which he detected in the seeds. Mercadante (1875) and Cossa (1875) noted that the decrease of asparagine in maturing seedlings coincided with an increase in malic and succinic acids, deposited largely as calcium salts. They suggested on this rather slight evidence that asparagine was deaminated in the plant, as in fermentation or *in vitro*, to the dicarboxylic acids.

Pfeffer (1872) held that formation of asparagine in germination was an oxidative process. He deduced that its regeneration to protein required a supply of carbon, presumably from carbohydrate, as in asparagine each nitrogen atom is associated with two carbons, the ratio in protein being about four. Asparagine was considered to arise in protein breakdown and to transport nitrogen from the cotyledons to growing points in the seedling. These ideas came mainly from

microscopic observation of asparagine crystals in tissues treated with alcohol. Borodin (1878) applied the same method to developing dormant buds, which physiologically resembled germinating seeds. Some buds (e.g. *Spiraea sorbifolia*) had much asparagine, some (e.g. *Quercus pedunculata*) a little, and others (e.g. *Alnus glutinosa*) none. Its formation was induced, or increased where it already occurred, by depletion of carbohydrate reserves. Borodin concluded that, in the presence of carbohydrate, asparagine was used in protein synthesis; in carbohydrate deficiency it accumulated. He also put forward the then highly speculative idea that respiration in plant tissues is associated with continuous synthesis and breakdown of protein. This concept, now widely supported, then had little experimental backing except the observation (Garreau, 1851a, b; Corenwinder, 1878) that young plant organs, with high protein contents, respire intensely.

Schulze (1878) found that seedlings of *Lupinus luteus* grown in the dark with no external nitrogen supply contained amino-acids and peptones as well as asparagine. Amino-acids detected in germinating seedlings included leucine (von Gorup-Besanez, 1874a; Cossa, 1875), tyrosine (Schulze & Barbieri, 1877), phenylalanine (Schulze & Barbieri, 1879), valine (Schulze & Barbieri, 1883), arginine (Schulze & Steiger, 1880), histidine and lysine (Schulze, 1878). Palladin (1888) showed that seedlings germinating anaerobically formed no asparagine; leucine and tyrosine accumulated. Godlewski (1903) and Suzuki (1900-02b) made similar observations. The presence of free amino-acids in seedlings suggested that in germination protein broke down to products resembling those of hydrolysis *in vitro*. Green (1887) reported that a proteolytic enzyme from *Lupinus hirsutus* formed leucine, tyrosine, and asparagine from seed protein of the same species. The substrate being dialysed, the asparagine probably came from asparaginyl residues in the protein, though the author did not clearly state this. Amide residues exist in seed proteins (see Chapter 7).

Leguminous seedlings show particularly striking accumulations of asparagine, but it was found by Schulze in other species, including *Papaver somniferum* (Papaveraceae), *Pinus sylvestris* (Coniferae), and *Tropaeolum majus* (Tropaeolaceae). Some species, e.g. *Cucurbita pepo*, *Helianthus annuus*, and *Linum usitatissimum*, form asparagine and glutamine in comparable amounts (Schwab, 1936; Vickery & Pucher, 1943). In others glutamine predominates, especially in the families Caryophyllaceae, Chenopodiaceae, Cruciferae, and Umbelliferae (Schulze, 1896b).

Prianishnikov (1895, 1899a, b, 1900, 1904) made extensive studies on the relation of asparagine to the breakdown and regeneration of protein in seedlings. He confirmed that asparagine arose largely by secondary processes from amino-acids, the primary products of protein breakdown. In contrast to Schulze, he considered amino-acids better suited to protein synthesis than asparagine, whose main function was to store in harmless form ammonia produced in the respiration of amino-acids. He noted that asparagine and soluble carbohydrate could occur together in plant organs without protein synthesis, and attributed accumulation of asparagine to metabolic inertness rather than to activity. Prianishnikov (1952) summarized in an excellent book the work of his school in relation to other studies on nitrogen metabolism in plants.

Suzuki (1897) demonstrated the synthesis, in plants removed from the soil to culture solutions containing urea or ammonium salts, of asparagine, which he deduced was formed from ammonia and a non-nitrogenous precursor, either carbohydrate or some substance closely related to it metabolically. Prianishnikov & Shulov (1910) compared barley seedlings grown in distilled water and in a culture solution with ammonium chloride. Supply of ammonia had no effect on the protein content per seedling, but markedly increased the asparagine content; the increase in free ammonia was very small. Pea seedlings grew badly in the ammoniacal solution used for barley, but addition of calcium sulphate improved growth and increased asparagine synthesis. Asparagine formation was here dissociated from protein breakdown, arising from ammonia supplied externally and from carbon furnished by the

TABLE 9

*Effect of carbohydrate and of light on the formation of asparagine in seedlings (Prianishnikov, 1924).*

<i>Experimental conditions</i>		<i>Results</i>	
<i>Carbohydrate supplied</i>	<i>Light</i>	<i>Asparagine synthesized</i>	<i>Ammonia accumulated</i>
+	—	+	—
—	—	—	+
+	+	+	—
—	+	—	+

reserves of the seed. Beetroot similarly forms glutamine when supplied with ammonia (Vickery, Pucher, & Clark, 1936)

Prianishnikov (1913, 1922a, b) and Smirnov (1923) studied the relations between ammonia and amides in seedlings of varied physiological types. Barley seedlings, with substantial reserves of carbohydrate in the seed, continue for a long time to form asparagine when absorbing ammonium salts in the dark, as do pea seedlings supplied with calcium. Seedlings of *Lupinus luteus* form little asparagine in the dark even if supplied with calcium, absorbed ammonia accumulates as such. In this species asparagine formation requires a concurrent supply of carbohydrate, coming from photosynthesis or supplied externally to plants grown in the dark. The effects of light and of external carbohydrate supply are shown (Prianishnikov, 1924) in a diagram (Table 9)

### C. Asparagine and Glutamine in detached Leaves

Borodin (1878) detected asparagine by the microchemical method in green leaves (*Lathyrus odoratus*, *Lupinus* spp., *Vicia cracca*, *V. sativa*) only after they had been held for several days in the dark in a moist atmosphere. Schulze & Bosshard (1885), using more quantitative methods, found some asparagine in normal leaves of *Acer pseudoplatanus*, *Platanus orientalis*, and *Trifolium pratense*, they showed also that protein decreased and asparagine increased in detached shoots (*Betula alba*, *Populus nigra*, *Vitis vinifera*) stood in water. Similar losses of protein and gains of asparagine occurred in darkened plants of *Avena sativa* and *Vicia faba* (Schulze & Kisser, 1889, Butkevich, 1908). Schulze (1895) isolated glutamine from detached leaves of *Beta vulgaris* and plants of *Saponaria officinalis* held in the dark. Kiesel (1906) found arginine, histidine, leucine, and valine in darkened plants of *Trifolium pratense*.

Protein generally decreases rapidly in detached leaves, with soluble nitrogenous compounds increasing at the same time. Miyachi (1897) followed protein breakdown and asparagine accumulation in detached leaves (*Paeonia albiflora*, *Camellia thea*). He showed that leaves on the plant contained over 90 per cent of their nitrogen as protein, a few days after picking almost half the nitrogen was in soluble form, asparagine being prominent in each species. Similar observations are recorded for many species, e.g. barley (*Hordeum sativum*) (Yemm, 1937, McKee, 1950), Sudan grass (*Andropogon sudanense*) and Kikuyu grass (*Pennisetum clandestinum*) (Wood, Cruickshank, & Kuchel, 1943, Wood, Mercer, & Pedlow, 1944), *Vicia faba* (Mothes, 1926), *Phaseolus multi*

*florus* (Chibnall, 1924a, b; Mothes, 1926; Moyse, 1950), rhubarb (*Rheum raphaniticum*) (Ruhland & Wetzel, 1927; Vickery, Pucher, Leavenworth, & Wakeman, 1938), *Rumex acetosa*, *Polygonum fagopyrum* (Moyse, 1950). Ribonucleic acid also breaks down, with accumulation of inorganic phosphate, in detached tobacco leaves (Ryzhikov & Gorodskaya, 1950). In detached vine leaves (*Vitis vinifera*) Deleano (1912) found no change in protein content for five days. Stability of protein in detached leaves is unusual, though young leaves of *Atropa belladonna* maintained their protein for three days (James, 1949). Most workers have used leaves of mesophytic plants; little is known about nitrogen metabolism in detached sclerophyllous leaves. The net loss of protein in detached leaves may mask continued synthesis, as estimates of total protein represent only the algebraic sum of opposed catabolic and anabolic processes. Net increases in protein in detached leaves have been recorded (*Helianthus*, Zaleski, 1897; *Narcissus pseudo-narcissus*, Pearsall & Billimoria, 1937, 1939; cotton (*Gossypium*), Phillis & Mason, 1942b; *Cichorium intybus*, Deken-Grenson, 1954). Studies with labelled nutrients detected some protein synthesis in detached leaves showing a net loss of protein (Andreyeva & Plyshevskaya, 1952; Chibnall & Wiltshire, 1954; Racusen & Aronoff, 1954). Axelrod & Jagendorf (1951) found that in detached tobacco leaves the soluble cytoplasmic protein fell by about 45 per cent in seven days, but there was no corresponding decrease in the activity of invertase, peroxidase, or phosphatase. They concluded that the proteins of these enzymes were not involved in the general breakdown. Other explanations are also possible, enzymatic activity being sensitive to many factors besides the amount of enzymatic protein present. Nitrogen from proteins broken down in detached leaves appears in amino-acids and particularly in amides. Absolute losses of nitrogen have been reported in detached leaves (Pearson & Billimoria, 1937) but are not usually found. After long starvation leaves lose some nitrogen as gaseous ammonia (Yemm, 1937; McKee, 1950), but at this stage they may be invaded by micro-organisms (Charles, 1954).

The carbohydrate and protein metabolism of detached barley leaves has been studied (Yemm, 1935, 1937, 1950; McKee, 1950) in relation to their respiration. The respiration rate was high immediately after the leaves were removed from the plant, fell rapidly for about 48 hours, and then remained steady at a lower level or rose again to give a characteristic two-humped time-curve. Carbohydrate was rapidly depleted, particularly sucrose, the main reserve sugar; the contents of fructose,

fructosan, and starch also fell, but there was a temporary accumulation of glucose. Over the first 24 hours the respiratory carbon dioxide was roughly equivalent to the loss of carbohydrate; later the carbon dioxide produced exceeded the equivalent of the carbohydrate lost. This indicated utilization of other substrates, probably the carbon skeletons of amino-acids produced by protein hydrolysis.

Protein breakdown began within a few hours after detachment of the leaf, being marked even in the early period when carbohydrate appeared to be the only substrate of respiration. Glutamine accumulated at first, decreasing later while asparagine accumulated, as Mothes (1940) also found in detached leaves and darkened seedlings of several species. The content of amino-acids rose steeply over the first 48 hours and then declined slowly. The accumulated asparagine finally broke down with liberation of ammonia; death of the leaf cells probably occurred at this stage. Asparagine and glutamine both accumulated in greater amounts than could have arisen directly in proteolysis, and were presumably formed from aspartic and glutamic acids produced by transamination.

Protein breakdown in detached leaves is largely independent of their carbohydrate content. Krotkov (1939) found little difference in the times when "secondary substrate materials", presumably including protein, first acted as important respiratory substrates in detached wheat leaves varying widely in initial sugar content. Vickery, Pucher, Wakeman, & Leavenworth (1937) analysed detached mature leaves of tobacco supplied with water and held in the light or the dark. In the light photosynthesis increased the carbohydrate content, but over the first 72 hours protein broke down at the same rate in the light as in the dark; later protein breakdown was considerably greater in the dark.

Wood and his co-workers (Wood, Cruickshank, & Kuchel, 1943; Wood, Mercer, & Pedlow, 1944; Wood & Cruickshank, 1944; Cruickshank & Wood, 1945; Wood & Womersley, 1946) presented very extensive and detailed data on metabolic changes in detached leaves of several grasses (*Andropogon sudanense*, *Avena sterilis*, *Pennisetum clandestinum*). Numerous individual constituents were estimated, including amino-acids, amides, betaine, choline, and organic acids. Leaves of *P. clandestinum* lost carbohydrate as rapidly in nitrogen as in air, but the protein content was unchanged over long periods. Chlorophyll, chloroplast protein, and ascorbic acid all decreased at similar rates in air but were stable in nitrogen for long periods. It was suggested that in normal conditions chlorophyll, protein, ascorbic acid and other constituents of chloroplasts exist as a complex in which



protein is inaccessible to proteolytic enzymes. In air this complex was assumed to be broken down by oxidation, being replaced in the attached leaf by continuous synthesis of protein. Injured leaves lost protein in nitrogen, forming amino-acids but not amides.

The amino-acids formed by proteolysis were metabolized at varying rates. The most rapidly used were cystine, glutamic acid, arginine, tyrosine, and tryptophan, in that order. Aspartic acid and some other amino-acids accumulated in greater amounts than could have been produced by proteolysis and must have arisen secondarily, their nitrogen at least presumably coming from other products of protein hydrolysis. Betaine, choline, and purines showed little change during starvation in these leaves.

Wood and his co-workers deduced from their results the following metabolic sequence:

(1) One or more amino-acids, including cystine, are oxidatively deaminated, forming ammonia and non-nitrogenous substances at a rate dependent on the sucrose content. (2) Sulphur-rich protein, including chloroplast protein, is hydrolysed to restore equilibrium among the amino-acids. (3) Glutamine is formed from ammonia produced by (1) and glutamic acid produced in (2); also from ammonia and  $\alpha$ -ketoglutaric acid arising in respiration. (4) Asparagine is formed from ammonia and aspartic acid arising directly and indirectly from protein hydrolysis. (5) Citric acid is formed from pyruvic acid (arising in glycolysis) and oxalacetic acid or malic acid at a rate determined by the contents of sucrose and oxalacetic acid. (6) Oxalacetic acid is formed from aspartic acid, or by oxidation of citric acid or  $\alpha$ -ketoglutaric acid. Malic acid is produced in equilibrium with oxalacetic acid. With a falling rate of respiration more  $\alpha$ -ketoglutaric acid is formed from glutamic acid. Malic acid and oxalacetic acid increase by oxidation of  $\alpha$ -ketoglutaric acid; aspartic acid, formed by transamination of other amino-acids with oxalacetic acid combines with ammonia to form asparagine.

#### D. Metabolic relations between Asparagine and Glutamine

The similar metabolic behaviour of these amides led early workers to assume that they were interchangeable, one or other fulfilling a general "amide" rôle in different species. It now appears, however, that both amides are generally distributed, their functions in the plant being somewhat different. Asparagine often seems to store ammonia in excess of immediate requirements for the synthesis of amino-acids, as in

plants receiving excessive external supplies of ammonia, or respiring the carbon skeletons of amino-acids in carbohydrate deficiency. Amino-acids and particularly amides often accumulate if protein synthesis is reduced or prevented by deficiency of essential mineral elements. This occurs in deficiencies of potassium (harley, Richards & Templeman, 1936; Richards & Berner, 1954; pineapple (*Ananas*), Sidris & Young, 1946a), sulphur (tomato, Nighthingale, 1932; sunflower, Eaton, 1941; lucerne (alfalfa), Mertz & Matsumoto, 1956), magnesium (tobacco, Steinberg, Bowling, & McMurtrey, 1950), phosphorus (tomato, McGillivray, 1927; oats, Richards & Templeman, 1936; *Phalaris tuberosa*, Williams, 1938), copper (tung (*Aleurites fordii*), Gilhert, Sell, & Drosdoff, 1946), iron (pear, Bennett, 1945; *Macadamia*, Guest, 1943; *Hibiscus esculentus*, Démétriadès, 1955, 1956a, b; Démétriadès & Constantinou, 1956; *Beta vulgaris*, *Pisum sativum*, *Pteridium aquilinum*, De Kock & Morrison, 1958), zinc (oats, Wood & Sibly, 1952; tomato, Possingham, 1956) and chlorine (cabbage, cauliflower, Frency, Delwiche, & Johnson, 1959). Amides, particularly asparagine, accumulate in chlorotic iron-deficient leaves and also in chlorosis caused by virus infection (Laloraya & Rajarao, 1956; Laloraya, Varma, & Rajarao, 1956) or by failure to form chlorophyll in white parts of variegated leaves (Molliard, 1911b; Schumacher, 1928; Molliard, Échevin, & Brunel, 1938). Arginine accumulates in the white parts of variegated leaves of *Bougainvillea glabra* (De Kock & Morrison, 1958).

The response of individual amino-acids to different deficiencies is variable, even in a single species. Possingham (1956) compared the free amino-acids of tomato plants deficient in copper, iron, manganese, molybdenum, and zinc with those of normal plants. Total free amino-acids increased in all deficiencies except that of molybdenum. Deficiency of iron and zinc, but not of copper or manganese, led to accumulation of asparagine and glutamine.  $\beta$ -Alanine accumulated in deficiency of copper, molybdenum, or zinc, and pipercolic acid when iron or manganese was deficient; these amino-acids were not detected in normal tomato plants. Phenylalanine was not detected in copper-deficient plants, though present in all other cases. Kulayeva, Silina, & Kursanov (1957) found that in the pumpkin phosphorus deficiency decreased formation of alanine and  $\gamma$ -aminobutyric acid, both prominent constituents in normal plants, and increased the content of glutamine, arginine, and allantoin. Putrescine accumulated in potassium-deficient barley plants (Richards & Coleman, 1952).

Glutamine seems to be more reactive and more directly related to protein synthesis than asparagine. Steward & Street (1946) found a close association between the glutamine content of potato tubers in different physiological conditions and their synthesis of protein. Assimilation of external nitrogen supplies in seedlings of pea (Rautanen, 1948) and barley (Willis, 1951) led to rapid synthesis of glutamine. Kretovich, Yevstigneyeva, & Plyshevskaya (1956) found that sugar beet, lupin, and vetch incorporated  $N^{15}$ -labelled ammonia into amide and amino groups of both asparagine and glutamine, the rate of incorporation being considerably higher for glutamine than for asparagine. In both amides the amide group took up ammonia nitrogen more rapidly than the amino group. The picture is similar for yeast absorbing inorganic nitrogenous compounds (Roine, 1946; Yemm & Folkes, 1954).

Rijven (1955, 1956) found glutamine a better nitrogen source than asparagine for young embryos of several plants; in some species, e.g. *Capsella bursa-pastoris*, asparagine supplied alone inhibited growth except at concentrations below 10 mg/l. Glutamine is prominent in metabolically active organs, while asparagine accumulates mainly in conditions interrupting normal metabolism, as in senescent or detached leaves, and etiolated seedlings. In some plant tissues a high supply of ammonia causes rapid and massive synthesis of glutamine. The beetroot, for instance, on fertilization with ammonium sulphate forms much glutamine with no corresponding increase in asparagine (Vickery, Pucher, & Clark, 1936). Glutamine synthesized in response to an external supply of ammonia may be excreted in leaf exudates which on evaporation deposit a white crust of the amide, as observed in rye-grass (*Lolium perenne*) (Greenhill & Chibnall, 1934; Raleigh, 1946) and in *Achillea millefolium*, *Hieracium pratense*, and *Rumex acetosella* (Curtis, 1944). Naylor & Tolbert (1958) found that when  $C^{14}$ -labelled aspartic acid was supplied to the leaves of 16 species of plants isotopic carbon always accumulated more in glutamine than in asparagine. Kretovich & Yakovleva (1959) found glutamine and glutamic acid much more active metabolically in ripening ears of wheat than asparagine and aspartic acid. Champigny (1958a) supplied glutamic acid, labelled in various positions with  $C^{14}$ , to developing plants of *Bryophyllum daigremontianum*; labelled carbon appeared in the expected products glutamine,  $\gamma$ -aminobutyric acid and proline, and also in numerous compounds less obviously related to glutamic acid, which is clearly an active metabolite in this species also.

### E. Structural relationships between Asparagine and Glutamine

Glutamine is thus active metabolically, in contrast to asparagine which appears predominantly as a storage substance providing a reserve of less readily mobilized nitrogen. These metabolic differences between two substances whose generally accepted structural formulae

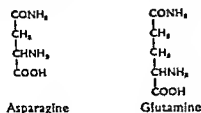


FIG. 44.

differ only by a single methylene ( $-\text{CH}_2-$ ) group (Fig. 44) have led to the suggestion (Steward & Thompson, 1952; Yevstigneyeva & Kretovich, 1953) that asparagine in solution has a cyclic structure (Fig. 45).

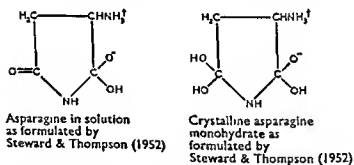


FIG. 45.

Differences between the two amides include the much greater solubility of glutamine in water; it is also highly labile to acid hydrolysis, a property utilized in the earlier methods for its determination in the presence of asparagine. Glutamine, unlike asparagine, is hydrolysed by boiling water. The amide and amino groups of glutamine both yield gaseous nitrogen on treatment with nitrous acid, but only the amino group of asparagine reacts in this way (Chibnall & Westall, 1932). Glutamine is also more active than asparagine in the formation of dark condensation products with xylose (Kretovich & Tokareva, 1948). Asparagine differs from glutamine and most other amino-acids in its reaction with ninhydrin (Ruhemann, 1911). Carbon

dioxide is liberated in the formation of the familiar purple colour when amino-acids react with ninhydrin. Asparagine gives a brown colour and yields no carbon dioxide if treated with ninhydrin in mild conditions; the purple colour is produced and carbon dioxide liberated on heating.

Steward & Thompson (1952) attributed to glutamine, which behaves similarly to other amino-acids, the accepted straight chain structure, and to asparagine the cyclic structure (amino-succinimide) shown in Fig. 45. Yevstigneyeva & Kretoich (1953) based somewhat similar views on a comparison of absorption spectra of the ninhydrin compounds. Glutamine, like other amino-acids, gave an absorption maximum at 570 m $\mu$  after treatment with ninhydrin; asparagine gave a quite different spectrum but, when it was heated, the peak at 570 m $\mu$  appeared. The Russian workers compared the absorption spectra of the ninhydrin compound of asparagine with that of proline, an imino acid giving a yellow colour with ninhydrin and possessing a cyclic structure somewhat resembling that proposed for asparagine. The ninhydrin compounds of proline and asparagine gave almost identical spectra, in agreement with a cyclic structure for asparagine. When asparagine and ninhydrin reacted in the absence of oxygen, the purple colour and the corresponding absorption peak at 570 m $\mu$  appeared at once, the cyclic form of asparagine apparently being stable only in the presence of oxygen.

The cyclic formula proposed for asparagine by Steward & Thompson (1952) has been criticized by various authors. Leach & Lindley (1953), from a study of hydrolysis rates, and Sidel (1953) from X-ray structural data for asparaginyl peptides and ultra-violet absorption spectra of the free amide, decided against the proposed structure. Saito, Cano-Corona, & Pepinsky (1955) also concluded from X-ray studies that in its crystalline monohydrate asparagine has an open chain structure. Sondheimer & Holley (1954) found aminosuccinimide to be distinguishable in solution from asparagine; it formed a brown compound with ninhydrin and combined with water at 37°C and pH7 to give a mixture of asparagine and isoasparagine. Katz, Pasternak, & Corey (1952) considered the configuration of asparagine in glycyl-L-asparagine incompatible with the aminosuccinimide structure. This structure thus seems untenable. The differences between the properties of asparagine and glutamine nevertheless seem excessive for homologous compounds differing only by a methylene group. The structure of asparagine, long believed to have been finally settled by Piutti (1887, 1888a), must still be considered uncertain.

## F. Comparative Biochemistry of Asparagine and Glutamine

These amides are unusual in that, although discovered and mainly studied in plants, they are now recognized as important animal metabolites. This situation is rare, animal biochemistry being on the whole more developed than that of plants.

### (i) Glutamine

Thierfelder & Sherwin (1914) showed that man excretes ingested phenylacetic acid as a conjugate with glutamine. Phenylacetylglutamine, now known as a normal constituent of human urine (Stein, Paladini, Hirs, & Moore, 1954), is synthesized in human tissues from glutamine and phenylacetyl Co enzyme A (Moldave & Meister, 1957). Glutamine is prominent among the free amino acids of many mammalian tissues (Ferdman, Frenkel, & Silakova, 1942; Hamilton, 1945; Stein & Moore, 1954; Tallan, Moore, & Stein, 1954). It is synthesized in tissues of mammals (Krebs, 1935; Speck, 1947) and birds (Ørstrøm, Ørstrøm, Krebs, & Eggleston, 1939). Ørstrøm (1941) found an active glutamine metabolism, apparently linked to glycolysis, in fertilized eggs of the sea urchin *Paracentrotus lividus*. Fertilization is followed by a large increase in the rate of ammonia uptake by the egg, the absorbed ammonia is stored as glutamine, synthesized from glutamic acid. Numerous studies (e.g. Bessman, Rossen, & Layne, 1953; Roberts & Bregoff, 1953; Kometiani & Klein, 1953, 1956; Vrba, 1955) show the great metabolic activity of glutamine, and the related compounds glutamic acid and  $\gamma$  aminobutyric acid in mammalian brain.

Glutamine is an essential growth factor for *Streptococcus haemolyticus*, it is very specific, glutamic acid and glutamyl peptides being unavailable (Mellwain, 1939; Mellwain, Fildes, Gladstone, & Knight, 1939). It is also required by *Lactobacillus arabinosus* (Hae, Snell, & Williams, 1945).

### (ii) Asparagine

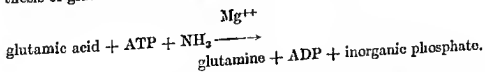
Several micro organisms appear to have a specific requirement for asparagine (Titum, Peterson & Fred, 1935; Niven, 1944; Wright & Skoger, 1944). Its metabolic relationships in these species are not, however, clearly understood.

Krebs (1935), finding a highly active asparaginase in some mammalian tissues, suggested that they might metabolize asparagine. Dietary asparagine is used by rats (Krotkov, Masoro, Nelson, & Reed, 1953).

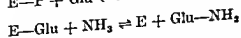
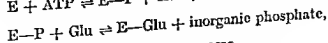
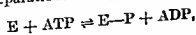
Free asparagine occurs in insects (Ussing, 1945; Kaplan, 1948), and in the crustaceans *Cancer pagurus* and *Homarus vulgaris* (Fraser, Kermack, Lees, & Wood, 1952). Mardashev & Semina (1950) isolated crystalline asparagine from liver; it is reported in other mammalian tissues (Krebs, 1950; Barry, 1953), including those of the cat (Tallan, Moore, & Stein, 1954) where it cannot arise from vegetable food. Animal proteins contain glutamyl and asparagyl residues, as in insulin (Chibnall & Rees, 1952; Sanger & Thompson, 1953a, b). Such residues occur in the polypeptide animal hormones oxytocin and vasopressin (Acher & Chauvet, 1953; du Vigneaud, Lawler, & Popenoe, 1953; du Vigneaud, Ressler, & Trippett, 1953; Tuppy, 1953; Lawler, Taylor, Swan, & du Vigneaud, 1954). These hormones also contain glycnamide, the free amide being unknown among natural products; glycnamide ribotide and its formyl derivative are, however, known as intermediates in purine synthesis in animals (Goldthwait, Peabody, & Greenberg, 1956a, b).

### G. Biochemistry of Amide Synthesis

Krebs (1935) showed that the synthesis of glutamine in animal tissues required oxygen and was inhibited by cyanide; he concluded that energy-yielding reactions were involved, as confirmed by later studies with cell-free systems (Speck, 1947; Frei & Leuthardt, 1949). The synthesis of glutamine from glutamic acid follows the equation:



This reaction occurs in preparations from *Staphylococcus aureus* (Elliott & Gale, 1948) and several higher plants (Elliott, 1951; Webster, 1953a, b, c; Dénes & Gazda, 1953; Kretovich, Yevstigneyeva, & Makarenko, 1954). Webster & Varner (1954a), using radio-active phosphorus ( $\text{P}^{32}$ ) in preparations from peas, found the intermediate stages:

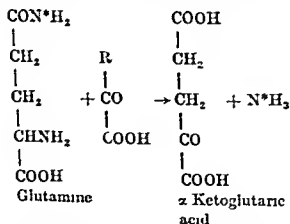


(E = enzyme; Glu = glutamic acid; Glu-NH<sub>2</sub> = glutamine).

The biosynthesis of asparagine has been studied in less detail than that of glutamine. Webster & Varner (1955b) found that in preparations from wheat and lupin its synthesis from ammonia and aspartic acid required adenosine triphosphate and was stimulated by magnesium ions. The concentrations of reactants required for synthesis in this system were, however, high enough to raise doubts regarding its significance *in vivo*. Yamamoto (1955) found that a similar synthesis of asparagine in germinating seedlings of *Vigna sesquipedalis* required adenosine triphosphate. Kretovich, Yevstigneyeva, & Makarenko (1954), working with etiolated shoots of lucerne (alfalfa, *Medicago sativa*) and pumpkin (*Cucurbita*) concluded that asparagine was synthesized from oxalacetic acid and ammonia in two stages catalysed by aspartase and asparaginase, a very different pathway from that observed in their material for synthesis of glutamine, which required ATP.

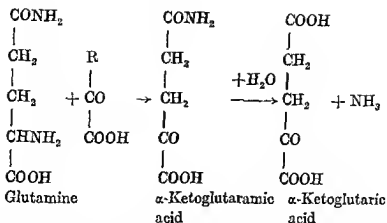
## H Transamination and Transamidation

Transamination between amides and keto acids has received much study in preparations from animal tissues. Meister & Tice (1950) showed that preparations from rat liver catalysed the following reactions between glutamine and a wide range of keto acids

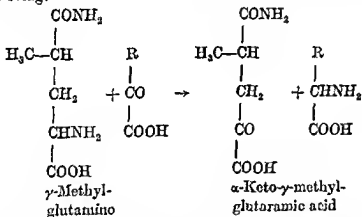


It was shown using  $\text{N}^{15}$  labelled glutamine that the ammonia liberated came from the amide group. Later work (Meister, 1953, 1954; Meister, Levintow, Greenfield & Abendschein, 1955) suggested that the reaction shown above occurred in two stages, each catalysed by a distinct enzyme.

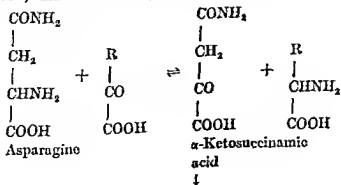


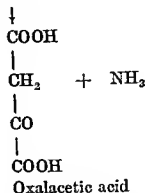


The substituted amides  $\gamma$ -methylglutamine and  $\gamma$ -methyleneglutamine were also active in transamination, but no ammonia was liberated during the reaction;  $\alpha$ -keto- $\gamma$ -methylglutaramic acid was isolated, the reaction being:



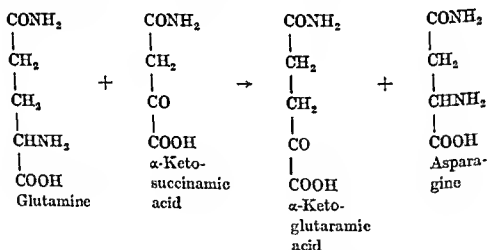
Transamination of asparagine in preparations from animal tissues is followed, as with glutamine, by deamination (Meister, Sober, Tico, & Fraser, 1952; Meister & Fraser, 1954):





The deamidation of  $\alpha$ -ketosuccinamic acid and  $\alpha$ -ketoglutaramic acid is catalysed by preparations from leaves (Meister, 1953).

The reversible conversion of asparagine to  $\alpha$ -ketosuccinamic acid offers a possible pathway for the synthesis of asparagine. No synthetic process forming  $\alpha$ -ketosuccinamic acid from simpler precursors is, however, known at present; in *Neurospora* it reacts enzymatically with glutamine to form asparagine and  $\alpha$ -ketoglutaramic acid (Monder & Meister, 1958):

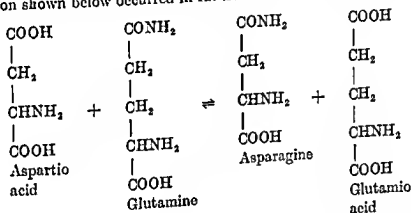


Wilson, King, & Burris (1954) showed that in various plant tissues asparagine transaminated with  $\alpha$ -ketoglutaric acid to form glutamic acid; Yamamoto (1955) also reported transamination between asparagine and pyruvic acid or  $\alpha$ -ketoglutaric acid in seedlings of *Vigna sesquipedalis*. Olenicheva (1955) detected in seedlings of soybean, pea, oats, and pumpkin, and in potato shoots, enzymes catalysing the transamination and deamidation of asparagine and glutamine. The ammonia liberated was transferred to glyoxylic acid, pyruvic acid, and phenylpyruvic acid, forming respectively glycine, alanine, and phenyl-

alanino. Activity of the transaminating and deamidating enzymes was greatly reduced in tissues of animals deficient in vitamin B<sub>6</sub>; this suggests pyridoxal phosphato as their co-enzyme.

Glutamino and asparagino are active in enzymatic (Meister *et al.*, 1952; Campbell, 1956) and non-enzymatic (Nakada & Weinhouse, 1953) transamination. The amides are, however, less susceptible than the corresponding dicarboxylic amino-acids to oxidative deamination (Mothes, 1940; Kretovich, Yevstigneyeva, & Makarenko, 1954). The interplay between amides and amino-acids may thus determine the manner in which ammonia or amino-groups are set free to take part in metabolic reactions.

Mardashev & Lestrovaya (1951) stated that the transamidation reaction shown below occurred in rat liver slices:



A similar reaction was reported (Sheffoer & Grabow, 1953) in yeast. Hsu (1959) could not detect transamidation in rat, rabbit, or pigeon liver, or in pigeon brain. Ammonia liberated by these tissues from asparagine was used in glutamine synthesis; the process occurred in two stages, not by direct transfer of amide groups as proposed by Mardashev & Lestrovaya (1951).

## I. Other Enzymatic reactions involving Amides

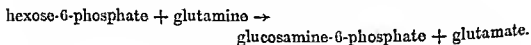
### (i) Deamidation

Lang (1904) showed that several animal tissues catalysed the removal of amide groups from asparagine and glutamine. Shibata (1904) found a deamidating enzyme in the mould *Aspergillus niger*. Similar deamidases are reported in yeast (Effront, 1908; Kurono, 1909b) and in *Penicillium camemberti* (Dox, 1909). The enzymes hydrolysing aspara-

gine and glutamine are apparently distinct; both are known from higher plants (Grover & Chibnall, 1927; Schwab, 1936; Steward & Street, 1946; Kretovich, Yevstigneyeva, & Makarenko, 1954; Yamamoto, 1955) but have not been studied in great detail. Germinating soybeans seem to use asparagine in forming ascorbic acid (Lee, Lee, Lee, & Kwon, 1959); both deamidation and deamination must be involved. A deamidase acting on  $\gamma$ -methylglutamine occurs in the peanut (*Arachis hypogaea*) (Fowden, 1955b).

(ii) *Synthetic reactions involving glutamine*

*Neurospora crassa* synthesizes the amino-sugar glucosamine by the following enzymatic reaction (Leloir & Cardini, 1953):



Glutamine is also involved in the synthesis of mucopolysaccharides formed from glucosamine in animal tissues (Boström, Rodén, & Vestermark, 1953), and of hyaluronate, also derived from glucosamine, in *Streptococcus* (Lowther & Rogers, 1955). Glucosamine is probably an important metabolite in fungi, being a precursor of chitin, their main structural constituent. Amino-sugars are widespread in plants and animals; they are recorded (Gladyshev, 1957) as constituents of a protein from soybean. Two diaminobexoses, a type of compound not previously known from natural products, occur in antibiotics (Rinehart, Woo, & Argoudelis, 1958).

In *Lactobacillus arabinosus* glutamine is required for the synthesis of arginine (Ory, Hood, & Lyman, 1954). It is also involved in the synthesis of histidine by *Escherichia coli* (Neidlo & Waelsch, 1956). Glycinamido ribotide and other intermediates in the biosynthesis of purines in animal tissues are formed by reactions in which glutamine participates. The reaction sequence has been formulated as follows (Goldthwait, 1956):

- (1) glutamine + 5-phosphoribosylpyrophosphate  $\rightarrow$   
5-phosphoribosylamino + glutamate,
- (2) 5-phosphoribosylamino + glycine + ATP  $\rightarrow$   
glycinamido ribotide + ADP,
- (3) glycinamido ribotide +  $C_4$  unit  $\rightarrow$  formylglycinamido ribotide.

This reaction sequence transfers from the amido group of glutamine the nitrogen atom that finally occupies position 9 of the purine nucleus.

The nitrogen atom at position 3 of this nucleus also comes from the amido group of glutamine, via the following enzymatic reactions (Levenberg & Buchanan, 1957*b*; Melnick & Buchanan, 1957):



- (4) formylglycinamide ribotide + glutamine + ATP  $\longrightarrow$  formylglycinamidine ribotide + glutamate + ADP,  
 (5) formylglycinamidine ribotide  $\rightarrow$  5-aminoimidazole ribotide.

5-Aminoimidazole ribotide is a precursor of inosinic acid (Levenberg & Buchanan, 1957*a*) and so of other purines.

These examples show the amide nitrogen of glutamine to be a very versatile participant in synthetic reactions. Interference with reactions involving glutamine has been invoked to explain metabolic inhibitions by the antibiotic azaserine, which is structurally similar to glutamine (Fig. 46). In some cases, e.g. preparations from pigeon liver (Hartman,

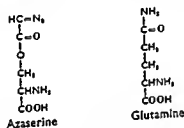


FIG. 46.

Lovenberg, & Buchanan, 1955), purine synthesis is an important site of azaserine action. The action of azaserine on *Gaffkya homari* (Aarensen, 1959) appears to be due to inhibition of some glutamine-requiring process other than purine synthesis. In the unicellular green alga *Scenedesmus* azaserine has little effect on the photosynthetic formation of sucrose; it causes, however, an accumulation of glutamine and of organic acids, suggesting an interference with transamination (Barker, Bassham, Calvin, & Quarek, 1956).

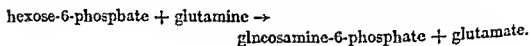
### (iii) Other exchange reactions of the amide group

Specific enzymes catalysing exchange of the amide group of glutamine with ammonia or hydroxylamine occur in the amoeba *Proteus vulgaris* (Waelsch *et al.*, 1950), in higher plants (Stumpf & Loomis, 1950) and in animals (Rudnick, Mela & Waelsch, 1954). The enzymes require manganous ions, phosphate or arsenate, and apparently

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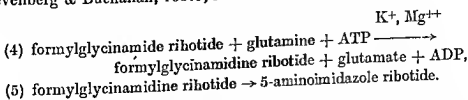
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- (2) 5-phosphoribosylamine + glycine + ATP  $\rightarrow$   
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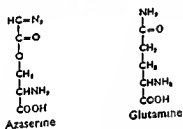


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adenosine diphosphate; arsenate, though unlikely to be a natural metabolite, gives greater activity than phosphate.

### J. The origin of Carbon Chains in Amides

Prianishnikov (1913, 1922a) established carbohydrate or its metabolic products as the non-nitrogenous precursors of amides. Malic acid, occurring widely in plants, was suggested as a close precursor of asparagine (Beyer, 1867; Prianishnikov & Shulov, 1910). Smirnov (1923) supplied maize seedlings with ammonium sulphate, malate, succinate, and aspartate. His results suggested some utilization of carbon from the organic acids, but were inconclusive owing to the long time required for the experiment and perhaps to poor absorption of substrates. Björkstén (1930) introduced the vacuum infiltration method, which fills the intercellular spaces of a leaf with a solution containing the substrates being tested, and brings them into close contact with active cells. If transpiration removes excess water promptly, air fills the intercellular spaces again. Protein synthesis continues and the tissue seems metabolically normal.

Mothes (1933) found that leaves of *Phaseolus multiflorus* infiltrated with solutions of ammonium aspartate, fumarate, malate, and succinate synthesized much more amide than control leaves infiltrated with water. He concluded that asparagine was formed, via aspartic acid, from fumaric, malic, and succinic acids. Schwab (1936) queried this conclusion, having found in infiltration experiments that amide formation seemed to be correlated with the supply of carbohydrate rather than of organic acids. Chihnull (1939) critically analysed the data of both authors, and concluded that the origin of the carbon chain of asparagine remained uncertain, particularly as organic acids present at the start of their experiments were not determined. He infiltrated leaves of perennial rye grass (*Lolium perenne*) with solutions of ammonium pyruvate and ammonium phosphate. In each case glutamine was rapidly synthesized; there was no change in the asparagine content. The very similar results with organic and inorganic ammonium salts showed that the leaves were well supplied with the non-nitrogenous precursor of glutamine, or formed it readily from available materials. Chihnull (1939) also infiltrated leaves of rye grass with a solution of ammonium  $\alpha$ -ketoglutarate. Most of the ammonia metabolized after intervals of 4 and 20 hours appeared as glutamine, organic acid being quantitatively utilized to form the carbon chain of the amide. Sugars disappearing during the experiment were probably used in respiration.



The respiration of leaves infiltrated with ammonium  $\alpha$ -ketoglutarate was greater than that of controls infiltrated with water; the difference may correspond to the energy used in the synthesis of glutamine from glutamic acid, a reaction known to be endothermic (Krebs, 1935). Kretovich, Bundel, & Gunar (1955) demonstrated the synthesis of glutamine from  $\alpha$ -ketoglutaric acid in pea seedlings, which also form aspartic acid from oxalacetic acid and ammonia (Kretovich, Bundel, & Aseyeva, 1951). Leaves of broad bean (*Vicia faba*) synthesize amides from the corresponding dicarboxylic amino-acids (Nelson & Krotkov, 1956).

Willis (1951) supplied ammonium phosphate labelled with  $N^{15}$  to detached roots from harley seedlings grown in conditions causing nitrogen deficiency and a high carbohydrate supply. The roots rapidly synthesized glutamine and to a lesser extent asparagine. Both amides contained  $N^{15}$ , showing that they had arisen from the external supply of ammonia; their synthesis was accompanied by a large increase in respiration rate.

The synthesis of aspartic and glutamic acids, and of their amides, is closely linked to other phases of metabolism. Their immediate non-nitrogenous precursors, oxalacetic acid and  $\alpha$ -ketoglutaric acid, are prominent members of the tricarboxylic acid cycle, a major pathway of oxidative carbohydrate breakdown in plant tissues, and take part in many other metabolic reactions. The dicarboxylic amino-acids, and their amides, also arise directly as products of protein hydrolysis, and indirectly from protein through transamination of other amino-acids. The metabolic situation in an intact tissue, as opposed to an isolated enzyme system, must therefore be highly complex.

## B. UREA AND UREIDES (ALLANTOIN AND ALLANTOIC ACID)

### A. Urea

Urea was long regarded as a specifically animal product, early plant physiologists (e.g. Boussingault, 1864, 1868) suggesting that in plants its metabolic function was taken over by asparagine. Urea was later found in fruiting bodies of *Lycoperdum gemmatum*, *Bovista nigrescens*, *Psalliota campestris*, and other higher fungi (Bamberger & Landsiedl, 1903; Goris & Mascré, 1908; Ivanov, 1923a, b, 1927), where it may accumulate to a remarkable extent, forming up to half the total nitrogen. In moulds and bacteria (Fosse, 1913a; Ivanov, 1925, 1926; Krebs

& Eggleston, 1939) urea arises by the hydrolytic breakdown of arginine coming from protein hydrolysis. In higher fungi it is also formed from carbon dioxide and ammonia (Ivanov, 1923b, 1927) and by an oxygen-requiring process from amino-acids (other than arginine) produced in protein hydrolysis (Ivanov, 1923c; Ivanov & Smirnova, 1928). Extracts from fungal fruiting bodies formed urea from arginine, but its synthesis from ammonia required intact tissues (Ivanov & Toshevikova, 1927). Kiesel (1927) suggested that in some fungi urea played the same metabolic role as in animals, converting to a harmless form ammonia arising by the breakdown of protein. Urea formed by fungi is not, however, usually excreted; it accumulates in developing fruit bodies but its nitrogen appears to be available for protein synthesis during spore formation (Ivanov, 1923a). It is not clear how urea is utilized in synthesis. One possible pathway is suggested by the presence (Ivanov & Ivetisova, 1931) of guanidinase in *Aspergillus niger*. This enzyme converts urea to guanidine, which in turn leads to arginine and other guanido compounds.

Fosse (1912) detected small amounts of urea in several higher plants, including *Brassica napus*, *B. oleracea*, *Cichorium endivia*, *Cucumis melo*, *Cucurbita maxima*, *Daucus carota*, and *Spinacia oleracea*. He pointed out that it was not necessarily a normal metabolite, but could have been absorbed as such from the soil. Later work (Fosse, 1913b) showed, however, that seedlings of these and various other species contained urea even when grown in water-culture to eliminate the possibility of it entering the plant through the roots. Fosse also introduced a sensitive and specific method of detecting urea as the dixanthyl derivative. Weyland (1912) found urea in the fern *Aspidium filix-mas* and the horsetails *Equisetum limosum*, *E. sylvaticum*, and *E. telmateia*, where he considered it to be associated with a copious development of endotrophic mycorrhiza in their roots; this was not confirmed by Weissfogel (1927). Other workers, e.g. Klein & Tauböck (1932a, b), Damodaran & Venkatesan (1948), Reiser & Melville (1949), have confirmed that urea is a widespread metabolite in higher green plants. Nevertheless, it remains uncertain whether free urea occurs in their tissues, except perhaps in very low concentrations. There is evidence (Fosse, 1926, Klein & Tauböck, 1932a, b, Damodaran & Venkatesan, 1948; Brunel, 1952; Mothes & Engelbrecht, 1956) that most of the urea in plant tissues is combined in labile ureides that break down to urea during analysis. Such ureides are presumably not attacked by urease, a widespread and active enzyme that would be expected to keep the level

of free urea very low in many plant tissues. Brunel (1952) examined 87 species of Leguminosae, once considered to be a characteristic urea-forming family, without detecting free urea; ureides were, however, often present, especially in the subfamily Papilionatae where they seemed more important metabolites than in the Mimosoideae and Caesalpinioideae.

### B. Allantoin and Allantoic Acid

Allantoin was first isolated from the amniotic fluid of the cow (Buniva & Vauquelin, 1800); plant sources included *Platanus orientalis* (Schulze & Barbieri, 1891), *Acer pseudoplatanus* and other woody species (Schulze & Bosshard, 1885), wheat germ (Richardson & Crampton, 1886), seeds of *Nicotiana tabacum* (Scurti & Perciaboso, 1906), and the root of *Symphytum officinale* (Titherley & Coppin, 1911). Later workers detected it in numerous other species. Allantoic acid, first recognized as a plant constituent in immature fruits of *Phaseolus vulgaris* (Fosse, 1920), has since been found in many species (Fosse, Brunel, & de Graeve, 1929a, b; Fosse, Brunel, de Graeve, Thomas, & Sarazin, 1930; Fosse, de Graeve, & Thomas, 1933), usually with allantoin but sometimes in its absence. Much of the evidence refers to seedlings, but allantoic acid occurs also (Leroux, 1937) in mature leaves of hazel (*Corylus avellana*, Betulaceae). The earlier work on allantoic acid and allantoin in plants has been well reviewed by Brunel & Capelle (1947). Reuter (1957a), in an extensive chromatographic survey, found ureides in many previously unexamined species. Most had one or two ureides; a few had three; *Acer pseudoplatanus* (Aceraceae) and *Parrotia persica* (Hamamelidaceae) had four. Individual ureides were not identified in this work. Ureides occur in ferns (Reuter, 1957a), mosses and liverworts (Touffet & Villeret, 1958), and in various green, brown, and red algae (Villeret, 1955, 1958; Sosa-Bourdouil, 1958). The ureides were accompanied by their associated enzymes, which also occurred in many species, particularly algae, where the substrates were not detected. Such species may also form ureides, though not accumulating them to detectable levels. Touffet & Villeret (1958) noted that, in contrast to other mosses, species of *Sphagnum* contained neither ureides nor the associated enzymes; this biochemical difference supports the view, based on morphological characters (Chaloud, 1945), that *Sphagnum* forms a quite separate group from other mosses.

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### C. Formation of Ureides and Urea in Plants

Three pathways leading to urea are known in plants:

- (1) arginine  $\rightarrow$  urea + ornithino;
- (2) canavanine  $\rightarrow$  urea + canalino;
- (3) purines  $\rightarrow$  allantoin  $\rightarrow$  allantoic acid  $\rightarrow$  urea + glyoxylate.

Only the third of these will be considered here as the others do not lead to the formation of ureides. In animals (Jones, 1904; Kerr & Seraidarian, 1945), higher plants (Schittenhelm, 1909; Kiesel, 1910), and yeast (Schutzenberger, 1874; Kossel, 1885) xanthine holds a central position in purine breakdown, other purines being converted to it before further catabolism. The conversion of adenine and guanine to xanthine involves deamination. Schittenhelm (1909) found an enzyme deaminating guanine to xanthine in lupin seedlings. *Azotobacter vinelandii* contains a specific adenine deaminase, which does not attack guanine or hypoxanthine (Heppel, Hurwitz, & Horecker, 1957). Individual purines are recorded from many plants. Kossel (1889) found xanthine and adenine in tea; Belzung (1892) showed xanthine to be abundant in seedlings of *Cicer arietinum*; Kiesel (1924b) obtained adenine, guanine, hypoxanthine, and xanthine from ripening ears of rye (*Secale cereale*). Methylated xanthines occur in various plants but are less widespread than xanthine itself; they are resistant to enzymatic breakdown, but appear to be metabolized before translocation from senescent leaves (Weevers, 1930). Tea (*Camellia thea*) contains theophylline (1,3-dimethylxanthine) and caffeine (1,3,7-trimethylxanthine); theobromine (3,7-dimethylxanthine) occurs in cocoa (*Theobroma cacao*).

In animal tissues xanthine is oxidized by xanthine oxidase to uric acid, a compound excreted by man and the higher apes, but in most other animals further oxidized by uricase to allantoin. Allantoin is converted by allantoinase to allantoic acid, split in turn by allantoicase to urea and glyoxylic acid (Fig. 47). All the compounds involved in this sequence have been found in plant tissues. The occurrence of xanthine, allantoin, and allantoic acid has already been mentioned. Uric acid, reported less frequently, is known from spores of *Aspergillus oryzae* (Sumi, 1928) and among higher plants from *Melilotus officinalis*, *Trifolium sativum*, and *Vicia faba* (Fosse, de Graeve, & Thomas, 1932a, b) and *Sorghum halepense* (Mikhlin & Ivanov, 1936).

The mode of action of uricase is still not entirely clear. There is evidence (Fischer & Ach, 1899; Behrend, 1904; Schuler & Reindel,

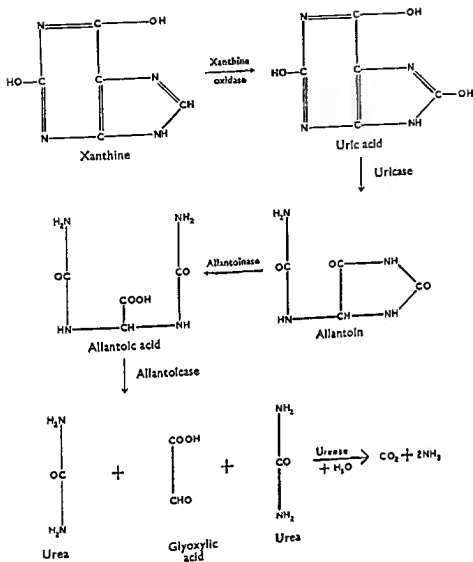


FIG. 47.

1932) that the first product of chemical oxidation of uric acid is a symmetrical compound, probably the compound (Fig. 48) usually known as hydroxyacetylenediuredocarboxylic acid (HDC); its correct systematic name is stated to be 5-hydroxy-3,7-diketo-2,4,6,8-tetra-azabicyclo[3,3,0]-octane-1-carboxylic acid (Bentley & Neuberger, 1952). Studies of the reaction between uric acid labelled with  $C^{14}$  and oxygen and water labelled with  $O^{18}$  suggest that HDC is also an intermediate in the enzymatic breakdown of uric acid (Bentley & Neuberger, 1952; Dalglish & Neuberger, 1954).

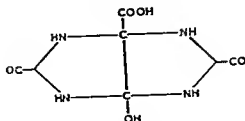


FIG. 48.

#### D. Enzymes of Purine Catabolism in Plants

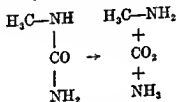
Xanthine oxidase, studied mainly in preparations of animal origin, is also known from moulds (Taha, Storek-Krieg, & Franke, 1955). Némec (1921) showed that soybean seeds formed ammonia from potassium urate and so probably contained uricase. Seeds of *Nicotiana tabacum* contain little uricase, but it is active in seedlings 2–3 weeks old (Gayrel, 1959). Fosse, Brunel, & de Graeve (1929a) found seeds of sixteen legumes to convert uric acid to allantoic acid. Ten of these seeds were known (Fosse & Brunel, 1929) to contain allantoinase; it was therefore assumed that a uricase formed allantoin which was then broken down to allantoic acid. Allantoicase has been found in the fungi *Aspergillus niger* and *A. phoenicis* (Brunel, 1939) and in some but not all of the legumes investigated (Échevin & Brunel, 1937a, b). Seeds of *Lupinus albus* (Échevin & Brunel, 1937a) and of *Agrostemma githago* (Brunel & Échevin, 1937) contain little or no allantoic acid, but it appears in appreciable amounts soon after germination. Villeret (1955, 1958) found allantoinase in numerous fresh-water algae, including *Chlamydomonas humicola*, *Chlorella pyrenoidosa*, *Staurastrum inflexum*, *Cosmarium formosulum*, *Zygnema circumcarinatum*, *Pleurochloris commutata*, *Nitzschia closterium*, *Anabaena cylindrica*, and *Calothrix parietina*. Allantoicase was detected in desmids only. Both enzymes were found in red, brown, and green marine algae, but allantoinase was less widespread than allantoinase. Touffet & Villeret (1958) studied twenty-five mosses and four liverworts. The levels of allantoin and allantoic acid were very variable in both groups. Mosses other than *Sphagnum* had much allantoinase and little allantoinase, the position being reversed in the liverworts. All the nine species of *Sphagnum* which were tested lacked both ureides and the corresponding enzymes.

Uricase is very specific, acting only on urea and on biuret ( $\text{H}_2\text{N.CO.NH.CO.NH}_2$ ) (Takeuchi, 1909; Shaw & Kistiakowsky, 1950). It was first discovered in bacterial extracts by Musculus (1876) but its existence was foreshadowed by Fourcroy & Vauquelin (1799) who



studied the conversion, presumably by bacterial action, of urea to ammonium carbonate in human urine on standing. They noted that this change did not occur if the urine were evaporated to dryness and the residue dissolved in water made up to the original volume. This procedure, they stated, destroyed "an albuminous or gelatinous animal substance acting as a ferment and responsible for the formation of ammonia". Urease is widespread in higher plants (Takeuchi, 1909; Kiesel, 1911; Zemplén, 1912; Fosse, 1914; Damodaran & Sivarama-krishnan, 1937; Brunel, 1952). Seeds are often good sources of the enzyme; the richest is *Canavalia ensiformis* (jack bean) (Annett, 1914). Other seeds with high urease activity occur in Leguminosae (e.g. *Dolichos biflorus*) and in Cucurbitaceae (e.g. the gourd *Trichosanthes anguina*, the giant pumpkin *Cucurbita maxima*, and the watermelon *Citrullus vulgaris*).

*Bacillus sphaericus* grows with *N*-monomethylurea as its sole source of carbon and nitrogen, metabolizing it by a reaction formally very similar to that catalysed by urease (Iyer & Kallio, 1958):



A molecule of methylamine thus replaces one of the ammonia molecules formed on hydrolysis of urea. The relation to urease of the enzyme catalysing this reaction is not clear.

#### E. Other pathways of Purine breakdown

Various other pathways occurring in bacteria are not known in higher plants. Barker (1943) showed that *Streptococcus allantoicus* formed oxamic acid ( $\text{HOOC}.\text{CONH}_2$ , oxalic semiamide) from allantoin. This substance has been found in sugar beet (Kminek, 1936) but nothing is known of its metabolism in higher plants.

The anaerobic breakdown of purines by *Clostridium acidu-urici* and *C. cylindrosporum* has been much studied. Here too other purines are attacked after conversion to xanthine (Radin & Barker, 1953; Rabinowitz & Barker, 1956b). Bacterial cultures produce carbon dioxide, ammonia, and acetic acid from xanthine and uric acid (Barker & Beck, 1941). In cell-free extracts the products are glycine (which in

intact bacteria gives rise to acetic acid), formic acid, and ammonia (Radin & Barker, 1953; Rabinowitz & Barker, 1956a). Ureidoimidazolyl carboxylic acid, aminoimidazole, and formiminoglycine have been identified as intermediates in the breakdown of xanthine (Rabinowitz & Pricer, 1956a, b; Rabinowitz, 1956). The breakdown of

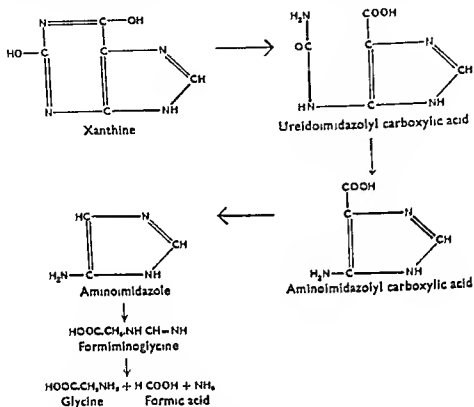


FIG. 49.

formiminoglycine to glycine, formic acid, and ammonia is an energy-yielding process in which adenosine triphosphate is formed by reactions involving folic acid (Rabinowitz & Pricer, 1956c). The main intermediates recognized in this sequence are shown in Fig. 49.

### F. Physiological functions of Ureides

Allantoin and allantoic acid, although less ubiquitous than the amides, are much more widespread as plant constituents than was formerly believed. In some species they play a major part in the storage and transport of nitrogen. Such species are often unrelated systematically, but ureido plants tend to be concentrated in some groups, notably the very important subfamily Papilionatae of Leguminosae. The earlier results of Fosse and his co-workers suggested, as did

those of Purucker (1932) with *Borago officinalis*, that the ureides were essentially products of purine catabolism. There is no doubt that they do arise in this way; later work, however, showed that some plants, e.g. *Acer pseudoplatanus*, *A. negundo*, *Wistaria sinensis* (Brunel & Échevin, 1938; Molliard, Échevin, & Brunel, 1938; Échevin, Brunel, & Sartorius, 1940), contained larger amounts of ureides than could arise in purine breakdown. Sosa-Bourdouil, Brunel, & Sosa (1941) found that in developing fruits and seeds of soybean allantoinic acid, and to a much smaller extent allantoin, were important transport compounds carrying nitrogen to the developing seeds or storing it temporarily in the hulls. A similar function for allantoin in other leguminous fruits was suggested earlier (Pfenninger, 1909; Schellenberg, 1916).

Mothes & Engelbrecht (1952b) found that allantoinic acid was the main nitrogenous compound in the bleeding sap of *Acer pseudoplatanus* and other species of the same genus. In these species it replaces the amides, which are present only in very small amounts, as a reserve of nitrogen for protein synthesis. The position is similar in *Symphytum officinale* (Mothes & Engelbrecht, 1954), where allantoin stored in the root system during the winter moves in the spring to the new growing shoots. In summer the roots contain little allantoin; its content increases sharply in autumn, when soluble nitrogenous compounds arising from protein breakdown in senescent leaves are translocated to the roots. The ureides are also major metabolites in some species where amides are prominent, e.g. *Phaseolus vulgaris* (Engelbrecht, 1954; Mothes & Engelbrecht, 1956). Allantoinic acid is important in the transport of nitrogen in *Persea americana* (Lauraceae), *Aesculus indica* (Hippocastanaceae), *Alectryon excelsum* (Sapindaceae), *Carica papaya* (Caricaceae), and *Cobaea scandens* (Polemoniaceae) (Bollard, 1957c).

Little is definitely known about ureide synthesis. Brunel & Brunel-Capelle (1951) reported an enzymatic synthesis of allantoinic acid in preparations of mushrooms (*Psalliota*). Conversion of allantoinic acid to allantoin was not found in these experiments. Roots of pumpkin (*Cucurbita*) supplied with  $C^{14}$ -labelled bicarbonate accumulate radioactive carbon in allantoin as well as in amino-acids. Alanine, normally the most prominent amino-acid, is replaced in phosphorus deficiency by allantoin, glutamine, and arginine (Kulaeva, Silina, & Kursanov, 1957).

Krupka & Towers (1958, 1959) found allantoin to be an active metabolite in germinating wheat seedlings, which contained little or no allantoinic acid. The roots formed allantoin much more actively than

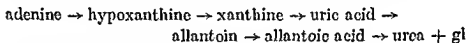
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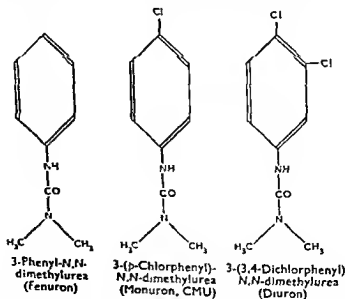
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the leaves. No evidence was found for its direct synthesis from glyoxylic acid. Glycine labelled with  $C^{14}$  was an effective precursor of allantoin, probably via purines. Seedlings supplied through the roots with uric acid or xanthine contained much more allantoin than those supplied with sucrose, or with sucrose plus ammonium nitrate. In wheat, allantoin thus appears to arise in purine breakdown; its catabolism leads to allantoic acid, which in turn forms glyoxylic acid; the latter is readily converted to glycine, a substrate for many synthetic reactions. Barnes (1959) showed that detached *Acer saccharinum* formed allantoin and allantoic acid from  $C^{14}$  adenine supplied through the petioles and suggested the catabolic sequence:



Other compounds related to urea occur in plants, but their catabolism remains obscure. Klein & Farkas (1930) isolated thiourea from seeds of *Laburnum anagyroides*. Ovcharov (1937) reported thiourea in healthy, and much larger amounts in rust-infected leaves of *Alchemilla vulgaris*, *Rhamnus cathartica*, and *Rubus saxatilis*. He found chlorophyll breakdown to be much accelerated in leaves treated with thiourea or their petioles in dilute solutions of thiourea compared with control leaves in water. The fungi *Botrytis cinerea*, *Pythium* sp., and *Vicia fabae* are also reported to produce thiourea.



*dahliae* are stated to form thiourea in culture (Ovcharov, 1937; Zelenin, 1939). Shantz & Steward (1955) identified a growth-promoting substance from coconut milk (*Cocos nucifera*) as 1,3 diphenylurea.

Some substituted ureas are powerful herbicides used in the complete removal of vegetation from industrial sites. They are also applied at low rates (of the order of 1 lb per acre or 1 kg per hectare) as selective pre-emergence weed-killers in various crops. The most-used compounds of this type are 3-phenyl-*N,N*-dimethylurea, 3 (*p* chlorophenyl)-*N,N*-dimethylurea, and 3 (3,4-dichlorophenyl)-*N,N*-dimethylurea (Fig. 50). The second of these, known as monuron or CMU, has received some physiological study. It enters roots easily, and is transported in the xylem to the leaves, where its main effects are localized (Muzik, Cruzado, & Loustalot, 1954). At very low concentrations in the leaf it specifically inhibits photosynthesis (Wessels & Van der Veen, 1956; Spikes, 1956).

## C. ARGININE AND CITRULLINE

### A. Arginine

Arginine, discovered in pumpkin seedlings by Schulze & Steiger (1886), is a regular component of proteins; some seed proteins, e.g. those of the pea (*Pisum sativum*) (Holmes, 1953), contain large amounts. The free amino-acid is widely distributed; it accumulates in seedlings of Leguminosae and Coniferae (Rongger, 1899; Schulze, 1904a), in tubers of cassava (*Manihot utilissima*, Euphorbiaceae) (Bigwood, Adriaens, & Médard, 1952; Close, Adriaens, Moore, & Bigwood, 1953), and in vegetative organs of numerous other species (Reuter, 1957a; Oland, 1959). It is prominent in immature pea seeds (Schulze, 1911; Spragg, 1955).

Arginase, which splits arginine to ornithine and urea, is widespread in flowering plants (Kiesel 1911, 1922b; Damodaran & Narayanan, 1940; James, 1949; Vaidyanathan & Giri, 1953) and in algae (Smith & Young, 1955). In animals arginine, together with citrulline and ornithine, takes part in a cyclic process forming urea (Krebs & Henseleit, 1932); there is good evidence (e.g. Skinner & Street, 1954; Kasting & Delwiche, 1955) for the occurrence of this cycle in higher plants also.

Arginine is thus clearly an active metabolite. It is less certain that it arises, like the amides, in response to high concentrations of ammonia in plant tissues. This possibility was suggested by Schulze (1896-97), who found large amounts of arginine in young seedlings of the conifers

*Abies pectinata*, *Picea excelsa*, and *Pinus sylvestris*. He concluded that part of the arginine arose by secondary transformations of the primary products of protein hydrolysis. His arguments, however, seem to assume a rather low arginine content in the seed proteins. Suzuki (1900-02a) claimed that in seedlings of *Cryptomeria japonica* and *Pinus thunbergii* arginine took the part played by asparagine in other seedlings, being formed on deamination of other amino-acids and in response to external supplies of ammonia. Schulze & Winterstein (1901) determined arginine in reserve seed proteins of several species. Seed proteins in the conifers *Picea excelsa*, *Pinus maritima*, and *Pinus sylvestris*, and in hemp (*Cannabis sativa*), were rich in arginine, suggesting that in their seedlings it could arise in quantity by protein hydrolysis. Schulze & Castoro (1904) showed that the arginine accumulating in etiolated seedlings of *Lupinus luteus* could all be formed directly in protein hydrolysis.

Mothes (1929), repeating Suzuki's experiments with seedlings of *Abies nordmanniana*, *Picea excelsa*, *Pinus nigra*, *Pinus pinea*, and *Pinus thunbergii*, found no secondary synthesis of arginine. Seedlings of *Picea* supplied with ammonia in the light or the dark formed asparagine rather than arginine, as was found also in *Pinus pinea* (Klein & Tauböck, 1932a, b). Guitton (1959) showed that in germinating seeds of *Pinus pinaster* arginase activity increased during imbibition much more rapidly than free arginine. Arginine was the main soluble nitrogen compound; asparagine and glutamine were also present, as in seedlings (Schulze, 1896-97) of *Picea* and *Pinus sylvestris*. It appears, as stressed by Mothes (1929), that protein hydrolysis accounts for accumulation of arginine in coniferous seedlings. In some species arginine is an important nitrogenous reserve, as in apple (Oland & Yemm, 1956), peach (Schneider, 1958), and *Phaseolus* (Pleshkov, Ivanko, & Antonova, 1957). Extraction of arginine in some experiments may have been incomplete; it is inefficiently extracted by 70 per cent ethanol, widely used as a solvent in such studies. Hot water, and sodium chloride solution buffered to pH 7 give better extraction (Oland & Yemm, 1956).

Arginine is the main free amino-acid in bulbs of tulip (*Tulipa gesneriana*); almost half the protein nitrogen of the bulb is in argininyI residues (Zacharius, Cathey, & Steward, 1957). The development of floral rudiments in the bulb is accompanied by amide formation at the expense of arginine. Arginine is typical of storage rather than active tissues in other species. It is abundant (Reuter, 1957a) in underground storage organs of *Allium ursinum*, *Anemone pulsatilla*, *Arum maculatum*,

*Macleaya cordata*, *Nymphaea alba*, *Polypodium aureum*, and *Pteridium aquilinum*, but much less prominent than amides and amino-acids in their growing parts. Similar results are recorded for *Oxalis deppea* (Liss, 1958).

## B. Citrulline

This amino-acid (see Chapter 7) is an important metabolite in Betulaceae and the related family Juglandaceae. It is a major constituent (Bollard, 1957c) of the xylem sap in several species scattered through other families. Detached shoots of hazel (*Corylus avellana*, Betulaceae) formed much citrulline in response to an external supply of ammonia (Reuter, 1957b). In hazel and some other species citrulline is the main soluble compound storing and transporting nitrogen.

## D. $\gamma$ -METHYLENEGLUTAMINE

In the germinating peanut (*Arachis hypogaea*) this amide accumulates markedly (Fowden, 1954a), being formed secondarily from the hydrolysis products of reserve proteins. It occurs also in the bulb of the tulip (*Tulipa gesneriana*), where it seems a rather inactive metabolite (Fowden & Steward, 1957b, Zacharius, Cathey, & Steward, 1957). A higher analogue of glutamine (aminocarboxyvaleramide, "homoglutamine") has been synthesized (Abraham & Newton, 1954) but is not known from natural sources.

## E. ETHYLGLUTAMINE (THEANINE)

This amide is an active metabolite in leaves of the tea plant where it is the most abundant free amino-acid (Sakato, 1957).

## F. OTHER AMINO-ACIDS

In rice (*Oryza sativa*) little amide is formed in response to external ammonium supply. Malavolta (1957) found the same amounts of amide in rice plants grown with nitrate and with ammonium salts; in both cases practically all the amide was glutamine. Zsoldos (1957) also found the amide content of rice plants to be almost unaffected by increasing supplies of ammonium, which, however, led to a large synthesis of alanine in the roots and of tyrosine in the shoots; both shoots and roots accumulated peptides. Other individual amino-acids were unaffected by ammonium supply.



Other amino-acids are prominent in the metabolism of individual species, e.g. azetidine-2-carboxylic acid in *Convallaria majalis* and *Polygonatum multiflorum* (both Liliaceae) (Fowden & Bryant, 1958, 1959; Fowden, 1959a) and  $\delta$ -N-acetylornithine in numerous species of Fumariaceae (Reuter, 1957a). These compounds may well be formed secondarily from ammonia arising within the plant or supplied externally; experimental evidence on this point is, however, lacking.

## G. NEUTRALIZATION OF AMMONIA BY ORGANIC ACIDS

Production of free ammonia and of organic acid are correlated in some moulds (Wehmer, 1891; Butkevich, 1903, 1922a, b). Ruhland & Wetzel (1926, 1927, 1929) and Kultschev (1932) suggested that in plants with highly acid sap (e.g. *Begonia semperflorens*, *Rheum hybridum*) excess ammonia arising in protein catabolism is neutralized by organic acids. Some of these plants are remarkably acid; oxalic acid forms 20 per cent of the dry weight in *Begonia semperflorens*, whose sap has a pH of 1.3. Other plants with acid tissues include *Fagopyrum esculentum* (Moyse, 1950), *Rumex acetosa* (Moyse, 1950; Liss, 1958), *Oxalis deppei* (Schwarze, 1932; Liss, 1958), and *Begonia hispida* and *B. nelumbiifolium* (Liss, 1958). Garher (1935) found that "acid" plants responded to gaseous ammonia by forming ammonium salts of organic acids; "non-acid" plants formed amides.

Extensive studies on the metabolism of acids and nitrogenous compounds in rhuhrb (*Rheum raphaniticum*) failed to show any correlation between ammonia content (usually quite low) and acid formation (Culpepper & Caldwell, 1932; Pueher, Clark, & Vickery, 1937a, b; Vickery, Pueher, Wakeman, & Leavenworth, 1939). Glutamine was found in rhuhrb leaves in spite of their high acidity. It occurs in other acid tissues, e.g. apple fruits (which also contain asparagine) (Hulme, 1936; McKee & Urbach, 1953), orange fruits (Scurti & De Plato, 1908) and leaves of *Oxalis deppei* (Liss, 1958). It has been suggested that glutamine cannot be stable in acid tissues. The tissues analysed are, however, clearly heterogenous in acidity and chemical composition, as demonstrated for *Oxalis deppei* and *Rheum rhabarbarum* by Liss (1958). Even within single cells the acidity and composition of the vacuole and the cytoplasm are known to differ. Glutamine is fairly stable at room temperature at pH 1.9; in these conditions Liss (1958) found 10 per cent hydrolysis in 24 hours and 50 per cent in 168 hours.

Acid tissues form amides ammonium salts of organic acids and arginine. The factors determining the proportions in which these compounds are formed remain obscure. It is also not clear whether neutral ammonium salts have some toxicity or can accumulate without damaging the cell.

## CHAPTER 11

# PROTEINS AND THEIR SYNTHESIS

## A. COMPOSITION AND STRUCTURE OF PROTEINS

### A. Historical

The first materials largely composed of protein to be studied were casein (from cheese) and albumen (egg-white). The terms in use today for protein in some languages, e.g. *Eiweiss* in German and *byelok* in Russian, are direct translations of the Latin word *albumen*. Other languages, e.g. English and French, use 'protein' as a general term, reserving 'albumin' for a particular type of protein. Grew (1682) and Gaertner (1788) applied the word 'albumen' to materials in seeds which resembled egg white in physical properties, and noted that they nourished the developing embryo plant just as reserves in the egg supplied the growing chick. The term protein, derived from the Greek *πρωτεος* (first; most important), was introduced (Mulder, 1838, 1839, 1840) in a sense distinctly different from that now used. Mulder concluded from analyses of several animal proteins, including fibrin, egg albumin, and silk, that they contained an organic radical  $C_{40}H_{62}N_{10}O_{12}$  combined with varying amounts of phosphorus and sulphur. The idea of organic radicals was then new; its introduction (Wöhler & Liebig, 1832; Berzelius, 1832) in the course of studies on benzaldehyde and related compounds was a major advance in organic chemistry, formulating a whole series of related compounds in terms of a single radical. This radical was named benzoyl, benzaldehyde being written  $BzH_2$ , benzoic acid  $BzO_2$ , and so on. This success encouraged Mulder to apply the same method; he named his supposed radical 'protein', formulating egg albumin as  $Pr_{20}PS_2$  and blood albumin as  $Pr_{20}PS_4$ . These formulae, though of course untenable, have the merit that the complexity of protein structure is recognized by an assigned molecular weight of over 17,000. Defects in this pioneer attempt at a chemical description of proteins were soon pointed out (Liebig, 1840; Laskowski, 1846) and interest in the subject lapsed for many years. The conclusion that protein molecules were very large, compared with those of simple chemical structure, was confirmed by

the observation (Graham, 1861) that they were retained by parchment membranes through which many substances passed freely.

Protein was long supposed to be essentially an animal product, its occurrence in materials of vegetable origin being considered anomalous. Osborne (1924), summarizing the history of investigations on plant proteins, could nevertheless cite several early students, beginning with Beccari in 1728, who obtained from plant sources substances that they recognized as similar to casein and other protein-rich animal materials. Beccari isolated from wheat grain the substance now called gluten, and noted that, in agreement with animal materials but unlike other plant products, it gave an alkaline distillate on destructive distillation. Kessel-Meyer in 1759 and Parmentier in 1773-76 also studied gluten, the latter recording its disappearance during germination. Rouelle (1773) obtained protein preparations by fractional heat coagulation of the juice of hemlock (*Conium maculatum*); one fraction contained nearly all the green pigment of the juice, another fraction was colourless and coagulated at a higher temperature. Fourcroy (1789) prepared similar materials from other plants. The proteins thus shown to exist in leaves received little further study for over 100 years. Vauquelin (1799) analysed the latex of *Carica papaya* and found that it contained a substance resembling blood albumin and showing all the properties of animal substances, in particular the formation of ammonium carbonate on destructive distillation. This observation, together with earlier data on albumins in leaves, led him to stress that plants as well as animals produce the compounds now known as proteins. Proteins are, however, more prominent in animals, where they are an important structural material, than in plants, which are built largely of substances derived from carbohydrate. In both groups metabolically active cellular material consists largely of protein. Braconnot (1813) noted that a fungus (*Boletus juglandis*) contained protein.

The difficulty of detecting any but the largest differences between individual proteins by proximate analysis delayed recognition of their great diversity. The individuality of certain proteins was admitted, but their number was believed to be quite small. Liebig (1841), for instance, stated that albumin, casein, and fibrin had the same composition, and saw little difference between plant and animal proteins. Even at this stage, however, some workers maintained that distinct proteins could be distinguished by chemical methods. Dumas & Cahours (1842), using a new and accurate analytical method, established comparatively large variations in the nitrogen content of proteins.

Their method is still used for reference work, though replaced for routine purposes by that of Kjeldahl (1883) and its many modifications. Norton (1848), working in Mulder's laboratory at Utrecht, Holland, analysed proteins from the seeds of almonds, oats, and peas, and concluded that the legumin of peas showed some striking points of difference from the other two proteins. Difficulties in obtaining pure preparations of individual proteins may well have been the limiting factor at this time rather than deficiencies of analytical technique.

The early workers knew that some amino-acids appeared on acid hydrolysis of proteins. Braconnot (1820) obtained glycine by acid hydrolysis of gelatine. He was aware that wood gave sugar on hydrolysis, and considered glycine (which has a sweet taste, the name being derived from the Greek *γλυκύς*) as 'sugar of gelatine'. He also used the name leucine for a product of protein hydrolysis, though it is improbable that his product was an even approximately pure specimen of the amino-acid now known by this name. Later (Braconnot, 1827a), in the course of a study on the toughening of peas cooked in hard water, he gave the name legumin, which is still in use, to a protein from pea seeds and recorded that on acid hydrolysis it formed 'leucine'. No attempt to distinguish between proteins by differences in their amino-acid content was made at this stage, nor would it have been a very profitable approach with the analytical methods then available. The first serious comparison of the amino-acids of different proteins was probably that made by Ritthausen (1872). Although he established large differences between proteins in the content of aspartic and particularly glutamic acids, Ritthausen concluded from his very extensive studies of seed proteins between 1860 and 1899 that the number of distinct substances of this class was comparatively small. Ritthausen laid the foundation for the chemical study of proteins; his work was extended by Osborne, who entered this field in about 1890 and summarized his results 30 years later (Osborne, 1924). In contrast to Ritthausen, Osborne stressed the great variety of different proteins and established that most, and perhaps all, of the species he investigated had quite distinct seed proteins.

Subsequent work has further emphasized the diversity of proteins, both by recognition of numerous enzymatic proteins and improved physical methods of characterization. It is now realized that proteins occur naturally in complex mixtures, whose resolution into their individual components may be extremely difficult. Accurate deter-

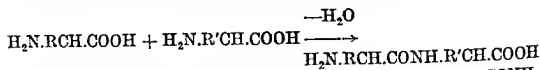
mination of the amino-acid residues of a protein specifies its composition far more precisely than is possible by elementary analysis; physical methods—diffusion, electrophoresis, measurement of osmotic pressure, sedimentation in the ultracentrifuge—establish homogeneity of particle size within fairly narrow limits. It is, however, impossible to establish finally by the methods now available that two proteins of different origin are identical, with the possible exception of proteins of low molecular weight where the sequence and arrangement of amino-acid residues can be unequivocally determined. The crystallization of proteins has encouraged undue faith in their homogeneity. Protein crystals have long been known. Hartig (1855) observed crystals of excelsin, a reserve protein in seeds of the Brazil nut (*Bertholletia excelsa*, Lecythidaceae), it was crystallized artificially by Maschke (1858). Many proteins, including numerous enzymes, have been crystallized but some are known to be heterogeneous even after repeated recrystallization. The  $\beta$ -lactoglobulin of milk, long cited as an outstanding example of a pure and homogeneous protein, is now known to contain distinct components, which remain together even after nine recrystallizations; it is an open question whether these newly separated constituents are themselves homogeneous (Smithies, 1954; Ogston & Tilley, 1955; Ogston & Tombs, 1957). Crystalline ribonuclease has also been separated into two enzymatically active components (Martin & Porter, 1951).  $\beta$ -Lactoglobulin was probably the first protein to be assigned an empirical formula ( $C_{1664}H_{3012}O_{576}N_{463}S_{21}$ ) with plausible claims to correctness. This formula was based on a considerable feat of amino-acid analysis (Brand, Saidel, Goldwater, Kassel, & Ryan, 1945); unfortunately the material used is unlikely, in view of later work, to have been homogeneous.

## B. Protein structure

### (i) Peptide linkages

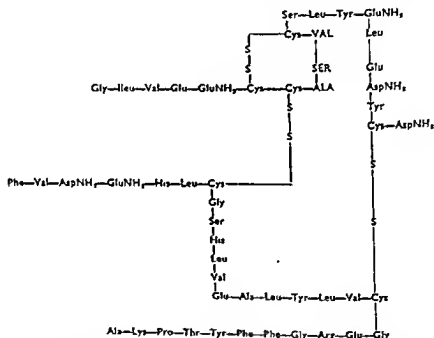
It was realized by 1900 that a considerable part, and possibly all, of the protein molecule was built up from amino-acid residues. The first clear suggestion on the nature of their linkage in protein was the polypeptide hypothesis, put forward independently by Fischer (1902b) and Hofmeister (1902). This hypothesis assumes that amino acids condense to form peptides, as in the reaction shown below, repeated condensation of peptides forming larger molecules of the same type and eventually protein. Any open-chain peptide, however many amino-

acid residues it may contain, must have at least one free amino group and one free carboxyl group available for further condensation.



The —CONH— or peptide linkage closely resembles the —CONH<sub>2</sub> group of amides.

Strong evidence that the peptide hypothesis represents the actual structure of protein came from studies on peptide synthesis from amino-acids (Fischer & Fourneau, 1901; Fischer, 1902*b*, 1906*a*; Curtius, 1904)



Structural unit of insulin (Amino-acid residues are shown by abbreviations of their names; GluNH<sub>2</sub> = Glutamine; AspNH<sub>2</sub> = Asparagine).

Structure is the same in all species investigated except in the part of the small ring shown in capital letters; here the sequence, reading downwards, is: beef, VAL—SER—ALA; Pig, whale, ILEU—SER—THR; sheep, VAL—GLY—ALA; horse, ILEU—GLY—THR. These three positions are cited in the text as A, B and C respectively.

FIG. 51.

and from the detection in protein hydrolysates of peptides varying in complexity from dipeptides to polypeptides containing ten or more amino-acid residues. Work of this type culminated in the determination of the complete structure of the insulin molecule (Fig. 51), one of the greatest triumphs yet achieved in the application of chemistry to the structural analysis of natural products. The unit structure of insulin

consists of two polypeptide chains one containing 21 and the other 30 amino acid residues they are joined by disulphide bridges between cysteinyl residues. Most of the amino acids commonly found in proteins occur in the insulin molecule. Aspartic acid, methionine and tryptophan are absent but the first of these occurs as asparagine. The terminal glycine and phenylalanine residues have free amino groups free carboxyl groups appear in the terminal alanine and asparagine residues (Sanger & Tuppy 1951a, b; Sanger & Thompson 1953a, b; Sanger, Thompson & Kitai 1955; Ryle, Sanger, Smith & Kitai 1955). The structure of beef insulin was first established later work (Brown, Sanger & Kitai 1955; Harris, Sanger & Naughton 1956) showed that insulins from sheep, horse and whale have small but distinct differences in amino acid composition affecting in each case the same sequence of three amino acid residues (Fig. 51). Pig insulin is identical with that from whale. These variants involve only replacement of amino acids by others that are structurally very similar: position A (Fig. 51) is always occupied by valine or isoleucine; position B by serine or glycine; and position C by alanine or threonine. The insulin units represented by these structures have molecular weights of about 6 000; the natural hormone probably contains two such units linked by an atom of zinc that joins the imidazole rings of the histidyl residues (Tanford & Epstein 1954). It is not clear whether the presence of zinc has any effect on the hormonal activity of insulin.

No regularity can be detected in the arrangement of amino acid residues in the polypeptide chains of insulin. Unit sequences are repeated in some proteins e.g. the sequence

(glycine-alanine-serine-glycine-alanine-glycine)<sub>7</sub> tyrosine

occurs in silk fibroin (Waldschmidt-Leitz & Zeiss 1955).

Much progress has already been reported towards the structural elucidation of ribonuclease (molecular weight 14 000) (Hirs, Stein & Moore 1956; Redfield & Anfinsen 1956; Ryle & Anfinsen 1957) and of lysozyme (molecular weight 14 700) (Fromageot & Privat de Garilhe 1949; Monier & Fromageot 1950; Thaureaux & Jolles 1956; Jolles, Thaureaux & Fromageot 1957; Jolles & Jolles 1958; Jollès, Jolles & Jauregui 1959). The first of these formidable studies in structural analysis was largely completed by the proposal (Hirs, Moore & Stein 1960) of a sequence for the 124 amino acid residues arranged in a single chain of ribonuclease. Anderer, Uhlig, Weber & Schramm (1960) put forward a sequence for the 157 amino acid residues forming the sub-unit



of tobacco mosaic virus protein. The structure of lysozyme also is almost completely established (Jollès, Jollès & Jauregui, 1960).

## (ii) Non-peptide linkages

The peptide linkage appears to dominate protein structure, but other types of linkage may occur in some proteins. This was stressed by Fischer (1906b), who suggested that diketopiperazine rings, and also linkages involving the hydroxyl groups of serine and tyrosine, might exist in proteins. Hydroxyl groups could, for instance, form ester links with free carboxyl groups of dicarboxylic amino-acids. The number of such ester groups is unlikely to be large, as in proteins with a high content of aspartic and glutamic acids the excess carboxyl groups are mostly in amide form.

Abderhalden (1923a) suggested diketopiperazine rings as the main units of protein structure; their occurrence in protein hydrolysates had

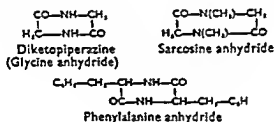
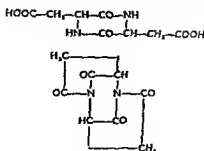


Fig. 52.

been recognized earlier. Bopp (1849) obtained leucinimide, subsequently shown to be a diketopiperazine. Structures involving this ring system (Fig. 52) were established for anhydrides of phenylalanine (Erlenmeyer & Lipp, 1883), sarcosine (Mylius, 1884), and glycine (Curtius & Schulz, 1890). The condensation of two molecules of aspartic acid, with elimination of two molecules of water, gives an anhydride with a diketopiperazine ring and two free carboxyl groups; elimination of two more molecules of water leads to another anhydride, probably of tricyclic structure, which has no carboxyl groups (Fig. 53). Glutamic acid forms similar derivatives (Ravenna, 1921; Blanchetière, 1924).

Convincing evidence that preformed diketopiperazine rings exist in the protein molecule is still lacking. Compounds with this ring have been isolated on partial hydrolysis of protein, but it is difficult or impossible to prove that they are not artefacts arising from amino-acids which in the intact protein formed polypeptide chains. Levene & Beatty (1906) isolated a polyglycyl anhydride from gelatine treated



Cyclic anhydrides of aspartic acid

Fig 53

with trypsin for 15 months. They avoided harsh methods of hydrolysis, but the gelatine was presumably prepared by the usual high temperature method. Abderhalden (1923b) boiled casein for two days in 5 per cent sulphuric acid and then held it at  $80^\circ\text{C}$  for four days in 10 per cent acid. The hydrolysate yielded a diketopiperazine containing leucyl and valyl

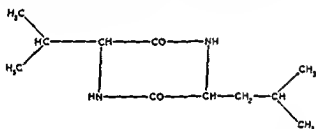
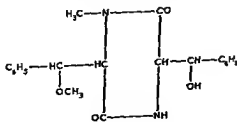


Fig 54

residues, but the treatment may have induced secondary ring formation. Sadikov & Lindquist Rysakova (1935) isolated a cyclic amino acid anhydride (Fig 54) from the hydrolysis products of blood albumin, but again the method of hydrolysis used suggests that it may have been an artefact. The existence of diketopiperazine rings in protein is at present neither excluded nor definitely demonstrated. In any case



Picrorocellin

Fig 55

they are unlikely to be quantitatively important in comparison with peptide linkages. One non-protein plant constituent, picrorocellin, isolated (Stenhouse & Groves, 1876) from the lichen *Rocella fuciformis*, is stated (Forster & Saville, 1922) to be a diketopiperazine derivative (Fig. 55).

Johnson & Burnham (1911) suggested the occurrence in proteins of thiopeptide linkages:



Polypeptides of this type were synthesized from glycine nitrile and hydrogen sulphide, one molecule of ammonia being eliminated for each thiopeptide linkage formed. They also proposed dithiopiperazine rings as structural elements in protein. The synthesis *in vitro* of these sulphur analogues of peptides is interesting, but there is no evidence that they occur in natural products.

Some proteins, e.g. myosin and tropomyosin from muscle (Bailey, 1951) and haemerythrin from the marine worm *Sipunculus nudus* (Holleman & Biserte, 1958), appear to have no terminal amino or carboxyl groups. If such groups are truly absent, not merely masked in some way from the agents used to detect them, the protein molecule must be cyclic in structure. Cyclic peptide structures may well occur in protein, as such peptides are known in fungi, e.g. phalloidine from *Amanita phalloides* (Šorm & Keil, 1951) and the antibiotics gramicidin-S (Sanger, 1946) and tyrocidin-B (King & Craig, 1955), and in higher plants (Eastwood, Hughes & Ritchie, 1955). Narita (1958a, b) isolated *N*-acetylseryltyrosine from chymotryptic digests of the protein from tobacco mosaic virus. In this protein the presence of terminal residues of *N*-acetylserine, rather than a cyclic structure, may be responsible for the absence of free amino groups.

There is some evidence (Bergmann & Miekeley, 1924; Blackburn, Middlebrook, & Phillips, 1942; Desnuelle & Casal, 1948) for oxazoline and thiazoline rings (Fig. 56) in proteins. Rings of this type are known

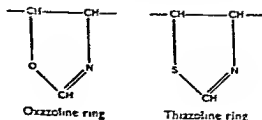
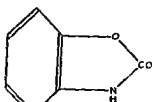


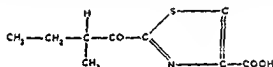
FIG. 56.



Benzoxazolinone

Fig 57

in a few natural products. Antifungal factors from seedlings of maize, rice, and wheat have been identified as benzoxazolinone and its 6-methoxy derivative (Fig 57) (Virtanen & Hietala 1955c, Hietala & Wahlroos 1956, Virtanen, Hietala & Wahlroos 1956). In the antibiotic bacitracin, cysteine and isoleucine are linked (Crug Hausmann & Weisiger 1954) to form a thiazoline ring, isolated as the thiazole carboxylic acid shown in Fig 58; a thiazolidine ring occurs in penicillin.



2-(1-Methylbutyryl)thiazole-4-carboxylic acid

Fig 58

Wrinch (1937a, b) proposed the cyclol structure, a meshwork of interlocking diazine and triazine rings, as the fundamental basis of the protein molecule. There is still no certain evidence that this structure occurs in protein; the alkaloid ergotamine, produced by the fungus *Claviceps purpurea*, has however a peptide portion containing a ring of this type (Fig 62) (Stoll & Hofmann 1950).

Abderhalden (1923a) suggested that disulphide bridges between cystinyl residues might be significant in protein structure. Such bridges occur in the insulin molecule; similar rings involving —S—S— bridges are found in the smaller peptide hormones oxytocin and vasopressin (Du Vigneaud, Lawler & Popenoe 1953; Du Vigneaud, Ressler & Trippett 1953). Sulphide bridges and other secondary bonds between polypeptide chains may be involved in the denaturation of proteins. This phenomenon was originally defined solely by changes in the properties of proteins; the causal changes in structure are not fully understood. Denatured proteins usually show reduced solubility at the isoelectric point and lack any enzymatic or hormonal properties.

possessed by the normal protein. Denaturation involves little if any change in the composition of a protein, but is accompanied by increased activity of side-chain groups in the molecule, such as the phenolic group of tyrosine and the disulphide group of cystine.

Denaturation is induced by varied insults to the protein molecule, including some much too mild to split peptide bonds. Heat, organic solvents, urea, anionic detergents, pressure, ultra-violet radiation, vibration, and pH values far to the acid or alkaline side of the isoelectric point all denature proteins, though proteins vary in sensitivity to these agents. Denaturation tends to increase the asymmetry of a protein, bringing the molecule to a state resembling the long straight peptide chain of the fibrous proteins rather than the compact structure of the globular proteins. It is now generally accepted that, as suggested by Wu (1931), denaturation results from the breaking of secondary bonds which in the normal protein bind together closely packed twisted or coiled peptide chains to form a definite three-dimensional structure whose geometry determines the properties of the molecule. On denaturation the precisely ordered structure is disorganized and the chains take up a random arrangement corresponding to a more stable thermodynamic state. Unfolding of peptide chains may expose to chemical action groupings previously held inaccessibly within the molecule, thus explaining the greater susceptibility to enzymatic hydrolysis noted for denatured proteins by various authors, e.g. Lin, Wu, & Chen (1928); Anson & Mirsky (1934); Haurowitz, Tunca, Schwerin, & Göksu (1945); Strachitski & Chernikov (1947); Huang & Niemann (1950).

The suggestion (Mirsky & Pauling, 1936) that hydrogen bonds are important in holding together the peptide chains of native proteins has been supported by later workers. Hydrogen bonds arise in protein when hydrogen atoms shared between the NH and CO groups of different peptide links form secondary links of the type:



These linkages are individually very weak, but their large numbers may help to maintain the fine structure of protein molecules in living tissues. Denaturation by such agencies as vibration shows that the bonds broken are weak; their lability is further indicated by the phenomena of protein spreading on a water surface. Proteins spread readily to form monolayers whose thickness corresponds to that of a single peptide chain; the secondary bonds between chains are thus

easily broken down during spreading Waugh, Wilhelmsen, Commerford, & Sackler (1953) concluded that in the formation of insubn fibrils interactions between secondary valencies of nonpolar side chains were more important than co valent, electrostatic or hydrogen bonds In this caso at least hydrogen bonds seem less important than other forces in maintaining protein structure

The spatial configuration of peptide chains has received much theoretical study, e.g. by Pauling, Corey, & Branson (1951), whose proposed structure for keratin and other fibrous proteins is consistent with the results of X-ray analysis (Perutz, 1951) Determination of the detailed structure of globular proteins, especially in the native or undenatured state, may require further progress in this difficult field

Dissociation of protein molecules into sub units bearing a simple relation to the size of the original molecule may be caused by processes similar to denaturation Concentrated solutions of urea split egg albumin and horse haemoglobin into fragments equivalent to half of the original molecule, edestin from hemp (*Cannabis sativa*) is similarly split into six equal fragments (Burk & Greenberg, 1930) Snail haemocyanin is split by urea and by ultra violet irradiation, giving fragments with one half, one eighth, and one sixteenth of the original molecular weight (Svedberg & Brohult, 1938) Krejci & Svedberg (1935) split wheat gliadin into two equal parts by heat or by adjustment of the pH Such dissociation makes it hard to define the true molecular weight of a protein The concept may indeed be misleading when applied to proteins The methods used to determine it measure properties, such as sedimentation rate or osmotic pressure, which depend on particle size The particles concerned may be molecular aggregates rather than individual molecules Some of the 'molecular weights' cited for proteins particularly nucleoproteins such as viruses, are extraordinarily high 'Molecular weights' of over 200 million are required by the sedimentation rates reported for bacteriophages (Sharp, Hook, Taylor, Beard, & Beard, 1946, Putnam, Kozloff, & Neil, 1949) Tobacco mosaic virus has a molecular (or particle) weight of 50 million (Williams, Backus, & Steere, 1951) and appears to contain about 3,400 terminal threonine residues (Harris & Knight, 1952)

Enzymes present in flour increase the solubility of wheat proteins without setting free any amino groups (Blagoveshchenski & Sossiedov, 1933, Blagoveshchenski & Yurgenson, 1935) These enzymes appear to disaggregate protein molecules without breaking peptide or other linkages between amino and carboxyl groups Possibly sulphide linkages are involved The cysteine content of proteins in flour is rather

low, but the few cysteinyl residues present might form bridges between peptide chains containing mainly other amino-acids.

### C. Conjugated Proteins

Numerous complexes of proteins with a wide range of other materials occur in plant and animal tissues. In some complexes protein is firmly bound to another substance (often called a prosthetic group) in stoichiometric proportions. Other protein complexes are of ill-defined composition and may be artefacts formed during isolation.

#### (i) *Protein-carbohydrate complexes (mucoproteins)*

Complexes of this type from animal sources usually contain amino-sugars, 2-aminoglucose or 2-aminogalactose; in mucoproteins of plant origin the polysaccharide appears to contain hexoses and pentoses but not amino-sugars.

#### (ii) *Lipoproteins*

Protein complexes containing large amounts of substances soluble in fat solvents occur in leaves, where they are often coloured with carotenoids and chlorophylls, and in seeds, where they are usually colourless. These complexes are often sufficiently stable to prevent direct extraction of the lipids by fat solvents.

#### (iii) *Nucleoproteins*

Compounds of proteins and nucleic acids are frequently reported, but it remains uncertain how many of them exist as such *in vivo*. Many of the nucleoproteins isolated from biological material are probably artefacts formed by combination of acidic groups of nucleic acids with free amino groups in protein molecules. Some nucleoproteins may, however, be definite chemical compounds, especially those of viruses.

#### (iv) *Haemoproteins*

Compounds in which protein is firmly bound to iron-porphyrin components are of great metabolic importance. The cytochromes form a group of respiratory pigments widely distributed among organisms; 80 per cent of the respiration of barley is mediated by the cytochrome system (James, 1953) and it is active in other plants and in bacteria, e.g. *Rhodospirillum rubrum* (Vernon & Kamen, 1954). Peroxidase and catalase are also conjugated proteins with iron porphyrins as prosthetic

groups The red pigment in the bacterial root nodules of Leguminosae is a haemoglobin (Kubo, 1939), one of a group of iron porphyrin respiratory pigments widely distributed in the animal kingdom but unusual in plants

(v) *Proteins with open chain tetrapyrrole prosthetic groups*

Chlorophyll and the haematin prosthetic groups of the cytochromes, haemoglobins and iron porphyrin enzymes contain a tetrapyrrole nucleus with the four pyrrole groups joined to form a ring The red algae

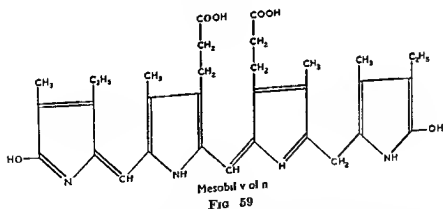
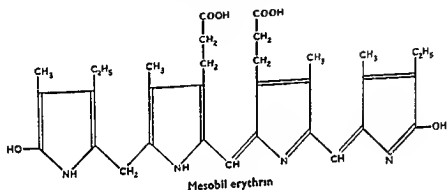


FIG 59

(Rhodophyceae) and blue green algae (Cyanophyceae) have auxiliary photosynthetic pigments formed of proteins combined with open chain tetrapyrroles related to the bile pigments. Phycocerythrin is considered typical of red algae and phycocyanin of blue green algae but both occur in each group. They can be separated by electrophoresis (Haglund & Tiselius 1950) or by chromatography (Krasnovski, Yevstigneyev, Brin & Gavrilova 1952). The prosthetic group of phycocerythrin is mesobilerythrin that of phycocyanin is mesobilviolin (Fig 59).



(Lemberg & Legge, 1949). These compounds are probably linked to protein by peptide bonds between their propionic acid side-chains and amino groups of the protein. Unusual or unknown amino-acids have been reported in phyocyanin and phyocerythrin by several workers (Wassink & Ragetti, 1952; Sisakyan, Bezinger, & Kivkutsan, 1954; Fujiwara, 1956) but the substances giving rise to these reports were probably peptides highly resistant to hydrolysis (Smith & Stockell, 1954; Kimmel & Smith, 1958).

#### (vi) *Flavoproteins*

Several enzymes from plants, e.g. diaphorase and the D-amino-acid oxidase of *Neurospora*, are flavoproteins with riboflavin phosphate or flavin adenine dinucleotide as the prosthetic group.

#### (vii) *Metal proteins*

Several enzymes contain a metal as an essential component. Well-known examples include copper in laeace (Keilin & Mann, 1939) and molybdenum in nitrate reductase (Nicholas & Nason, 1954a). Zinc forms a chelate compound with histidyl residues of insulin, but seems not to be required for its hormonal activity.

## B. PROTEINS FOUND IN PLANTS

### A. Types of Protein

Osborne (1924) divided proteins into albumins (soluble in water), globulins (soluble in aqueous salt solutions), glutelins (soluble in dilute aqueous alkali), and prolamins (soluble in 70-80 per cent ethanol, but insoluble in pure water or ethanol). This arbitrary classification is still widely used, though boundaries between the classes are not sharply defined. The distinction between albumins and globulins is particularly vague, partly because many proteins behave differently in solutions of different salts, and at different concentrations of the same salt.

The reserve proteins of seeds are better known than those of other plant parts. Many dicotyledonous seeds contain much globulin which after extraction with neutral salt solutions can be purified by dialysis or by fractional precipitation with ammonium sulphate. Oil-bearing seeds commonly contain well-defined globulins which crystallize readily. Osborne (1892) crystallized excelsin from the Brazil nut (*Bertholletia excelsa*) and also globulins from the seeds of *Cannabis sativa* (hemp), *Cucurbita maxima* (pumpkin), *Linum usitatissimum* (flax), and *Ricinus*

*communis* (castor-oil plant). The last seed contains ricin, an extremely toxic albumin studied by Osborne, Mendel, & Harris (1905), Kabat, Heidelberger, & Bezer (1947) and Kunitz & McDonald (1949). The lethal dose for mammals is stated to be 5g or less per kg of body weight. Ricin has been separated into two toxic proteins (Mourgne, Barct, Reynaud, & Bellini, 1958).

The molecular weights of seed globulins vary considerably, but in many cases (legumin from pea, arachin from peanut, amandin from almond, excelsin from Brazil nut, and cocosin from coconut) fall within the range 300,000 to 350,000 (Svedberg & Sjögren, 1930; Sjögren & Spychalski, 1930, Danielsson, 1949, Johnson & Shooter, 1950). In the pea seed Osborne & Harris (1907) found two globulins (legumin and vicilin) and an albumin (legumelin). Danielsson (1950b, 1952a) repeated this work and showed the globulin fractions to be heterogenous. Using other methods he obtained preparations appearing homogenous when tested in the ultracentrifuge and by electrophoresis. Their molecular weights were about 180,000 (vicilin) and 330,000 (legumin). Legumin was notably richer in tryptophan and in sulphur-containing amino-acids than vicilin. Danielsson (1952a) found similar globulins in seeds of many other legumes. Globulins from seeds of peanut (Johnson, Joubert, & Shooter, 1950) and lupin (Joubert, 1955) dissociate reversibly into smaller units. The albumin of pea seeds is highly heterogenous and contains various enzymes; it probably represents cytoplasmic protein from the embryo rather than a reserve.

In most cereals prolamins and glutelins occur in roughly equal amounts and form together about 80 per cent of the total protein. Globulins and albumins are quantitatively minor constituents but contain important enzymes. In barley  $\alpha$ -amylase appears to be a globulin and  $\beta$ -amylase an albumin (Äyräpää & Nihlén, 1954). Detection of enzymatic activity in seed proteins depends to an important extent on the method of extraction used. Kretovich, Bundel, Melik-Sarkisyan, & Stepanovich (1954) compared the enzymatic activity of proteins extracted from pea seeds by the method of Osborne in which the preparations are treated with organic solvents such as acetone, ethanol, or ether, and by a new method intended to avoid denaturation. In this method pea meal was extracted with 0.2 per cent sodium chloride solution, the filtered extract being dialysed against distilled water until all chloride was removed. The precipitated globulins were centrifuged off and legumelin was prepared by freeze-drying under vacuum. Freeze-drying was also used in the final preparation of the globulins. All

operations were carried out at temperatures near 0°C. Legumelin and vicilin prepared by the new method showed varied enzymatic activity (carboxylase, catalase, dipeptidase, glutamic dehydrogenase, invertase, and peroxidase); extracted by Osborne's method, legumelin had no enzymatic activity and vicilin slight activity of catalase and glutamic dehydrogenase only. Any protein for enzymatic studies must clearly be handled by gentle methods likely to avoid denaturation. The detailed results of Kretovich *et al.* (1954) conflict, however, with those of Danielsson (1950a), whose seed globulins prepared by apparently gentle methods had no enzymatic activity. The globulins of Kretovich and his associates were perhaps contaminated with enzymatically active albumins, or alternatively Danielsson's extraction procedure may have inactivated enzymes in his material.

Cereals with exceptional protein distributions include rice (*Oryza sativa*), which has little prolamins, almost all the reserve protein being glutelin, and oats, where it is mostly globulin. Among dicotyledonous seeds *Chenopodium quinoa*, used as grain in South America, has little globulin; *Plantago psyllium* contains over 80 per cent of its protein as glutelin.

### B. Amino-acid composition of Seed Proteins

The proteins of seeds contain most or all of the usual protein amino-acids, but in very variable proportions. Prolamins are distinguished by very high contents of glutamic acid, which contains about half the nitrogen of hordeins (barley) and avenins (oats). Most of the glutamic acid exists in glutamyl residues, the corresponding amount of ammonia being released on hydrolysis. Gliadins from wheat and rye and prolamins from *Agropyrum repens* had 37-44 per cent of their nitrogen in glutamyl residues; proline was the next most important amino-acid in all these prolamins (Reznichenko, Kolesov, Polotnova, & Chubachina, 1956; Kolesov, 1957). Most of the other amino-acids were present, including lysine and tryptophan, sometimes stated to be absent from prolamins, but none except glutamic acid and proline made a large contribution to the total nitrogen. Glutamic acid and proline predominated also in glutelins from barley, rye, and wheat, but less markedly than in prolamins (Waldschmidt-Leitz & Mindemann, 1957). The low content of aspartic acid contrasts in both types with the large amounts of glutamic acid. In globulins glutamic acid and arginine are the main amino-acids, aspartic acid and sometimes proline being other prominent constituents. The protein of sunflower (*Helianthus annuus*) has been

stated (Blagoveshchenski & Schubert, 1934) to contain over 14 per cent by weight of histidine. This very high histidine content is not confirmed by more recent analyses (Block & Bolling 1945; Edwards, Sealock, O'Donnell, Bartlett, Barclay, Tully, Tybout, Box & Murlin 1946), which agree in assigning to this protein a histidine content of about 2 per cent, as is usual in seed proteins. A high histidine content (10 per cent) is, however, reported for the protein of *Carthamus tinctorius*, another oilseed of the family Compositae (Babga, Rajagopalan, & Shivaramiah, 1954). The protein of *Ricinodendron rautanenii* (Euphorbiaceae), an important oilseed in Angola, is unusually rich in cystine and threonine (Adrian, Rerat & Xabregas, 1955).

### C The Proteins of Leaves

#### (i) *Extraction methods*

The presence of proteins in leaves was shown by early workers, but difficulties of extraction have impeded their study, and they are much less adequately known than seed proteins. Winterstein (1901) obtained protein preparations by drying leaves of various species (*Aesculus hippocastanum*, *Carpinus betulus*, *Lolium perenne*, *Lupinus albus*, *Medicago sativa*, *Spinacia oleracea*, *Trifolium pratense*) at a low temperature and extracting them with hot water. The preparations having 12 per cent or less of nitrogen presumably contained appreciable amounts of non-protein constituents. Osborne & Wakeman (1920) and Chubb & Schryver (1920) took up the problem independently. In each case leaves (spinach or cabbage) were ground in water and cellular debris removed by centrifuging. Chubb introduced an important technique, cytolysing the leaf cells with ether before grinding. Cytolysed leaves pressed before grinding yielded a liquid believed to represent the vacuolar contents. In spinach and lucerne (alfalfa) (Chubb & Nolan, 1924) and in watermelon (Kiesel, Belozersky, Agator, Bivshikh, & Pavlova, 1934) the liquid so obtained from leaves contained a little protein, in other species it was protein-free. The protein so obtained has been considered to exist in solution in the vacuoles of intact cells. The methods used do not, however, seem to preclude the possibility of its origin by leakage of cytoplasmic protein from damaged cells.

The residue after the cytolysed leaves had been pressed was ground in water, rupturing the cell walls and dispersing or dissolving the cell contents. The cell wall debris was removed by straining through silk gauze, chloroplasts and nuclei were filtered out using paper pulp, and

cytoplasmic protein was obtained by flocculation of the filtrate with acid. Two main fractions, corresponding roughly to chloroplastic and cytoplasmic protein, were thus available for study. Many of the preparations had low nitrogen contents owing to the presence of non-protein constituents, particularly pentosans, which could be separated only with difficulty. Others consisted essentially of protein but were obtained only in low yields. Partial analyses suggested a similar amino-acid composition for the cytoplasmic and chloroplastic proteins; both groups are, however, likely to be highly heterogeneous, in view of the many different enzymes known to exist both in the chloroplasts and in the cytoplasm. Alkaline media, e.g. borate buffer at pH 9.2, extract from leaves almost all their protein, which can be precipitated from solution by heat or by acid (Lugg, 1939; Lugg & Weller, 1944). In this method the alkaline extractant should protect cytoplasmic proteins against alteration by the acid vacuolar sap.

The colloid mill has been used to disintegrate leaves before extraction of protein (Wildman & Bonner, 1947; Wildman, Campbell, & Bonner, 1949; Singer, Eggman, Campbell, & Wildman, 1952). In the leaves of seven dicotyledons (*Cucumis anguria*, *Lycopersicum esculentum*, *Nicotiana glutinosa*, *N. tabacum*, *Pisum sativum*, *Spinacia oleracea*, and *Xanthium pennsylvanicum*) an apparently homogeneous protein of high molecular weight formed 25 to 50 per cent of the total cytoplasmic protein. The association of purines, pentoses, and phosphorus with this material suggested that it was a nucleoprotein. It was a phosphatase but had no other enzymatic activity. It yielded small amounts of auxin on alkaline hydrolysis, and was therefore described as an auxin complex, but it is possible (Schocken, 1949) that the auxin found arose by the action of alkali on tryptophanyl residues in the protein.

#### (ii) *The proteins of chloroplasts*

Maschke (1859) showed by staining tests that proteins remained in plastids depigmented with acetone. In the method of Granick (1938) for the isolation of chloroplasts, leaves are ground in hypertonic or isotonic sucrose solutions. The grinding is as gentle as possible, but has to break cell walls to release chloroplasts from the cells. The chloroplastic protein is probably contaminated to some extent with that of cellular particles such as mitochondria. These can be separated from intact chloroplasts by differential centrifugation, but in ground material the chloroplasts are largely broken down to fragments comparable to mitochondria in size. These fragments may correspond to the grana,

pigmented structures known from morphological studies with the electron microscope to occur embedded in the colourless matrix or stroma of the chloroplast. The chloroplasts contain 30 to 45 per cent of the total protein of the leaf in several species of monocotyledons and dicotyledons. Protein forms 40 to 50 per cent of the dry weight in chloroplasts. Lipids form 20 to 40 per cent; they include chlorophyll which accounts for 4 to 8 per cent (Granick 1938, Menke 1938, Neish 1939, Hanson 1941, Hanson, Barrien & Wood 1941, Bot 1942, Comar 1942, Yemm & Folkes 1953). The presence of most of the usual protein amino acids and of hydroxyproline in chloroplast protein is reported by Sisakyan, Bezinger & Kuvayeva (1951); methionine, sulphoxide and  $\gamma$ -aminobutyric acid were also detected by paper chromatography in the hydrolysates but the authors considered them to be artefacts absent from the original protein.

Yemm & Folkes (1953) analysed preparations from barley containing (a) whole protein from mature leaves, (b) cytoplasmic protein from mature leaves, (c) whole proteins from seedlings. Very little difference was found between the amino acids from mature and seedling leaves except that the latter had slightly more lysine. Eighteen protein amino acids plus amide accounted for 96 to 98 per cent of the total nitrogen of the protein; hydroxyproline was not detected. The proteins had comparatively high contents of the basic amino acids arginine and lysine. Sisakyan, Bezinger, Gumilevskaya & Lukyanova (1955) recorded the partial amino acid composition of chloroplasts from very young, mature and senescent leaves of sugar beet. The proportion of the individual amino acids (expressed as a percentage of the dry weight of the plastids) showed rather little variation during the life history of the leaf; the contents of alanine and aspartic acid tended to fall with increasing age. High contents of arginine and lysine were found in this material also. Leucoplasts were also sampled from sugar beet roots at two stages of development. Their protein again showed high contents of arginine and lysine but differed from the chloroplast proteins in containing less of the dicarboxylic amino acids and more serine. Serine decreased and threonine increased with the age of the root supplying the leucoplasts; the total amount of these hydroxyamino acids remained almost unchanged, suggesting that threonine might be formed directly from serine.

Sisakyan, Melik-Sarkisyan & Bezinger (1952) and Sisakyan & Melik-Sarkisyan (1956) separated the protein complex from sugar beet chloroplasts into four components by electrophoresis and fractional

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Sisakyan, Melik-Sarkisyan, & Bezingier (1952) and Sisakyan & Melik-Sarkisyan (1956) separated the protein complex from sugar-beet chloroplasts into four components by electrophoresis and fractional



precipitation with different concentrations of ammonium sulphate. Two of the components were nucleoproteins containing ribonucleic acid which on hydrolysis yielded the purines adenine, cytosine, guanine, and uracil. The other two constituents were globulins with little nucleic acid. The stroma of the chloroplast is stated to contain only ribonucleic acid, in contrast to the grana, which have both ribonucleic acid and deoxyribonucleic acid (Metzner, 1952).

(iii) *Linkages between proteins and lipids in the chloroplast*

Stokes (1864) separated two green and two yellow pigments from green leaves; the yellow pigments (carotene and xanthophyll) are now known to be groups of related substances rather than individual chemical entities. All these pigments are intimately associated with protein in the chloroplast. Several early workers (e.g. Hoppe-Seyler, 1879, 1881; Reinke, 1886) pointed out that extracted chlorophyll differed from the green material of the leaf and was probably combined chemically with protein *in vivo*. Lubimenko (1921) noted that benzene, in which chlorophyll is very soluble, failed to extract the green pigment of dried leaves; he deduced that solvents that extracted chlorophyll directly broke some chemical bond linking it to protein. Further evidence for chemical combination between chlorophyll and protein in higher plants is cited by Baas Beeking & Hanson (1937), Smith (1941), and Griffith, Valleeau, & Jeffrey (1944); a similar association is also reported in photosynthetic bacteria (French, 1940). Godner & Osipova (1947) suggested that the tertiary nitrogen atoms of the pyrrole rings in chlorophyll combined with free carboxyl groups in protein. This suggestion is supported by the observation (Osipova, 1947) that proteins with an excess of carboxyl groups (gliadin and zein) absorbed 12 per cent of their weight of chlorophyll from its solution in petroleum ether; other proteins with few or no free carboxyl groups absorbed less than 1 per cent in the same conditions. Walkin & Schwartz (1953) suggested that chlorophyll molecules formed a monomolecular layer at an interface between protein and lipid components of the chloroplast, the porphyrin nuclei of chlorophyll being oriented towards the protein phase and the phytol side-chains towards the lipid phase. Takashima (1952) isolated from leaves of clover (*Trifolium repens*) a crystalline chlorophyll-lipoprotein complex containing for each molecular unit of protein (molecular weight 19,000) two molecules of chlorophyll. Sherratt & Evans (1954) obtained a similar complex from the green alga *Chlamydomonas dorsicentralis*. These complexes appear to be highly

labile, as in paper electrophoresis of the complex from spinach leaves the pigment does not follow the migrating protein component (Anderson, Spikes, & Lumry, 1954) to which it is bound only by weak adsorptive forces

Other lipid-soluble substances are concentrated in the chloroplasts; they contain, for instance, almost all of the vitamin E and vitamin K of the leaf (Dam, Glavind, & Nielsen, 1940). Similar associations are reported in animal material; Dzieduszyński, Mystkowski, & Stewart (1945) concluded from studies of solubility relationships and of the effects of denaturants, that in human blood plasma both carotene and vitamin A are combined with protein. Protein-lipid complexes may be expected to occur also in other intracellular structures, such as mitochondria, which contain substantial amounts of both constituents.

Numerous studies, e.g. by Michael (1935), Fagan & Ashton (1938), Smith & Wang (1941), Smith & Rohb (1943), Keirstead (1945), Sideris & Young (1947), have shown correlations between the contents of carotenoids, chlorophyll, and protein in leaves at varied stages of development and exposed to various environmental conditions. There are, however, well-known cases, such as ripening fruits of tomato (*Lycopersicum esculentum*) or persimmon (*Diospyros kaki*) and yellowing senescent leaves, where the carotenoids increase while chlorophyll decreases. Chlorophyll is always associated with carotenoids, but they occur without it in many flowers, fruits, and vegetative storage organs.

In grass leaves (Wood & Cruickshank, 1944) and in root tips of bean and onion (Randall, 1951) ascorbic acid may be combined with protein. The concentration of ascorbic acid in leaves varies much more than that of protein; leaves rich in this acid probably contain it largely in the free state.

## C. SITES OF PROTEIN SYNTHESIS IN THE PLANT

### A. General

Several early workers (see Chapter 2) held that amino acids were synthesized and condensed to protein mainly in the leaves and suggested that protein formation might require light in green plants. It was realized that light was not a general requirement, moulds being known to use nitrate in the dark as their sole source of nitrogen for growth and so presumably for protein synthesis. Later work showed that leaves (Zaleski, 1897) and roots (Postma, 1939) formed protein from nitrate

nitrogen in the dark if supplied with carbohydrate. Kinoshita (1897*a, b*), Suzuki (1898*b*), Mazé (1898*a*), and Maliniak (1900) also observed protein synthesis in the dark by plant organs.

## B. Protein Synthesis in Leaves

Chrapowitski (1887), Stoeck (1893), and Ullrich (1924) found that protein accumulated rapidly in nitrogen-deficient seedlings or detached leaves transferred to solutions containing nitrogen. The chloroplasts of starving leaves lose protein, suggesting that in normal conditions they store and probably synthesize protein. Plastids of non-green organs may also be associated with protein synthesis. Leucoplasts of sugar-beet roots form invertase (Sisakyan & Kobyakova, 1952); similar particles contain most of the protein in mature seeds of *Macadamia* (Proteaceae) (Francis, 1927).

Sapozhnikov (1894), Krashenninikov (1901), and Godlewski (1903) suggested that both protein and carbohydrate are formed in photosynthesis, a view strongly supported by later work. Burström (1943*a, b*) showed that in wheat leaves protein formation increased with rising light intensity and with assimilation of carbon dioxide. The distribution of isotopic carbon in unicellular algae and higher plants assimilating  $C^{14}$ -labelled carbon dioxide (Benson & Calvin, 1950; Nezgovorova, 1952, 1956; Tolbert & Zill, 1954) showed amino-acids to be formed in the first few seconds of photosynthesis. Alanine and aspartic acid were usually detected first, then glycine, glutamic acid, and  $\beta$ -alanine. Racusen & Aronoff (1954) found that darkening considerably reduced incorporation of  $C^{14}$  from labelled carbon dioxide into protein by soybean leaves; aromatic and branched-chain amino-acids were formed in the light only. Nezgovorova (1956) noted that high nitrogen supply greatly increased the formation of amino-acids from labelled carbon dioxide in *Phaseolus* leaves. Since the formation of other organic acids was unaffected, she suggested that amino-acids arose by carboxylation of aminated precursors. This seems to imply carboxylation of  $\beta$ -alanine, aspartic acid being the main radioactive amino-acid detected after 5 seconds exposure to labelled carbon dioxide. After 20 minutes exposure alanine, arginine, asparagine, glutamic acid, glycine, lysine, serine, and threonine contained isotopic carbon. Bidwell, Krotkov, & Reed (1954) found that much of the carbon assimilated by detached leaves (beet, tobacco) supplied with ammonium nitrate appeared in glutamine, a plausible precursor of protein. Kauffmann & Kosel (1959) found numerous oligopeptides in spinach chloroplasts; they may be

intermediates in protein formation from amino acids arising in photo synthesis

$N^{15}$  has also been used to study the effects of illumination on protein synthesis. Delwiche (1951) supplied immature tobacco leaves through the petioles with  $N^{15}$  labelled nitrate. Both in light and darkness isotopic nitrogen appeared in protein indicating that in this species light is not essential for protein formation even in the leaves. Andreyeva & Plysherskaya (1952) held leaves of *Nicotiana rustica* and *Zea mays* for 20 hours in solutions of  $N^{15}$  labelled ammonium sulphate. Batches of these leaves were then subjected to three experimental treatments, strong illumination with 1 per cent carbon dioxide, strong illumination in the absence of carbon dioxide, darkness in normal air. After four to six hours cytoplasmic and chloroplastic proteins were prepared from the leaves. Illuminated leaves supplied with carbon dioxide incorporated much isotopic nitrogen into chloroplast protein. Incorporation was less in light without carbon dioxide and negligible in the dark. Results for cytoplasmic protein were rather variable, in contrast to chloroplastic protein it showed in most experiments substantial synthesis in the dark. It thus appears that in leaves protein may be formed from inorganic nitrogen by two distinct pathways one being independent of light. Sulphur from  $S^{35}$  labelled sulphate and methionine appeared rapidly in chloroplastic and cytoplasmic protein of leaves from *Phaseolus* seedlings (Pleshkov & Ivanko, 1956). Sulphur supplied as sulphate appeared mainly in chloroplastic protein suggesting the plastids as a major site of sulphate reduction.

Protein synthesis and catabolism in leaves are strongly affected by substances transported from the roots. Chibnall (1954) found that protein broke down rapidly in the laminae of detached leaves of runner bean (*Phaseolus*) held with their petioles in water or damp sand. Non protein nitrogen was transferred to the petiole and chloroplasts degenerated in a few days. In leaves induced by auxin treatment to form roots protein breakdown in the laminae was greatly reduced and degeneration of the chloroplasts occurred only after six weeks. Mothes & Engelbrecht (1956) compared the metabolism of rooted leaves (*Nicotiana*, *Pelargonium*, *Phaseolus Symphytum*) with that of similar detached leaves without roots. Considerable breakdown of protein took place in detached *Phaseolus* leaves even under continuous illumination. The soluble nitrogenous compounds so formed were to a large extent translocated to the midrib, where protein synthesis occurred. These changes were retarded in leaves sprayed with solutions of ammonium

nitrate or of urea. Root formation had the same effect in the absence of any external supply of nitrogen, and its influence was more lasting. The nature of the essential constituents transmitted from the root to the leaf is not understood. The behaviour of rooted leaves could not be duplicated in isolated leaves supplied through the petioles with amino-acids, amides, protein hydrolysates, bleeding saps, or coconut milk. Old rooted leaves accumulated very large amounts of storage materials absorbed from the roots—nitrate and glutamine in *Nicotiana*, allantoin and allantoic acid in *Phaseolus*, allatoin and glutamine in *Symphytum*. Richmond & Lang (1957) showed that a supply of kinetin (6-furfuryl-aminopurine) greatly retarded the breakdown of protein and of chlorophyll in detached leaves of *Xanthium pennsylvanicum* (Compositae). The provision of kinetin from other parts of the plant may thus help to maintain the metabolic integrity of attached leaves; its mode of action is obscure, though it has marked effects on nitrogenous metabolism in detached leaves (Möthes, Eogelbrecht, & Kulayera, 1959) and on ribonucleic acid synthesis in roots (Guttman, 1957).

Comparison of the nitrogenous constituents of the green and white or yellow variegated leaves suggests that protein synthesis is much more efficient in the former. Church (1879) analysed white and green leaf tissue from *Alocasia macrorrhiza* and *Elaeagnus pungens*. In the former protein represented 34 per cent of the total nitrogen in white and 71 per cent in green tissue; the difference in *Elaeagnus* was less but in the same direction. Molliard (1911b) found a much higher proportion of soluble nitrogen in the yellow parts of variegated leaves of *Euonymus japonicus* than in the green parts. Lakon (1916) showed that in several variegated species (*Abutilon vexillarium*, *Acer negundo*, *A. pseudoplatanus*, *Aegopodium podagraria*, *Sambucus nigra*, *Tradescantia zebrina*, *Pinca major*) green tissues had much more protein than white. Yellow tissues, with plastids but no chlorophyll, had protein contents intermediate between those of white and green tissues. Schumacher (1929) observed that the ratio of soluble to protein nitrogen was much higher in white than in green tissues of leaves in *Acer negundo*, *Cornus albus*, *Peristrophe calicifolia*, and *Sambucus nigra*. The soluble nitrogen consisted largely of amino-acids and amides. Groner (1936) found three to five times as much amino nitrogen in albino seedlings of *Zea mays* as in green seedlings of the same age and strain. Molliard, Échevin, & Brunel (1938) also reported a high proportion of soluble nitrogen in white leaf tissue of *Acer negundo* (mainly allantoin and allantoic acid) and of *Pelargonium zonale* (mainly amino-acids and amides). Leaf

tissues with impaired capacity for photosynthesis are inefficient in protein synthesis also, though capable (Schumacher, 1928) of some synthesis if supplied with soluble carbohydrate. Chloroplasts are not the only site of protein synthesis even in green tissues. Microsomes play a major part in protein synthesis in animal tissues (Hoagland, Keller, & Zamcenik, 1956, Hoagland, Zamcenik & Stephenson, 1957), they may be equally important in this connexion in plants.

## C. Protein Synthesis in Seeds

### (1) General

Plant seeds vary greatly in size, structure, and physiological behaviour. Most orchids have tiny seeds, as do some dicotyledons: the average seed weight in *Nicotiana tabacum* is 0.08 mg. The largest familiar seed is probably the coconut (*Cocos nucifera*). Another palm, the double coconut or coco de mer (*Lodoicea maldivica*), has the largest known seed weighing 90 kg and taking 6 years to ripen (Good, 1951).

Some seeds retain the power of germination for centuries. Seeds of *Nelumbium nucifera* germinated after storage for 240 years as herbarium specimens (Anonymous, 1942), other seeds of this species germinated at ages not precisely known but perhaps as great as 1000 years (Ohga, 1926, Libby, 1951). *Albizia julibrissin* seed germinated 140 years after collection. Seeds of several other species, mostly Leguminosae, germinate after storage in ordinary conditions for more than 100 years. Germination does not occur until the hard impermeable seed coats, a barrier against uptake of water and perhaps oxygen, are broken artificially or by decay. Some weeds with permeable seed coats (e.g. *Rumex crispus*, *Oenothera biennis*) remain viable without germinating for 60 years in damp soil (Crocker, 1938) an inhibition of unknown nature preventing germination although the tissues are saturated with water. In contrast with such long-lived seeds, others germinate before the fruit is shed from the parent plant. This occurs regularly in *Sechium edule* (choko, chayote) and in several mangroves (*Avicennia*, *Rhizophora*) and is seen occasionally in oranges. Many seeds die within a few weeks of ripening, e.g. rubber (*Hevea brasiliensis*) and species of willow (*Salix*).

The embryo within the seed attains in different species very variable degrees of structural differentiation before its development is halted by the cessation of water supply from the parent plant. The tiny seeds of orchids consist of a few undifferentiated cells, in other seeds,

e.g. in various species of the families Cucurbitaceae, Gramineae, and Leguminosae, there is a well-developed embryo, with rudiments of stem and root, and sometimes of several leaves.

It is against this background of great diversity in structure and behaviour that we should consider the metabolism of ripening seeds. This has been studied for adequate and obvious reasons of economic importance and experimental convenience mainly with medium-sized seeds from the families Gramineae and Leguminosae. Scattered data are available for some other plants, but the detailed work in this field refers almost exclusively to cereals and pulses. Even in these groups the number of species studied is too small to permit any wide range of comparison.

## (ii) *Protein synthesis in leguminous seeds*

The rapid synthesis and accumulation of protein characteristic of ripening seeds are particularly striking in the familiar peas, beans, and pulses; large amounts of starch are laid down concurrently with protein, and some species, e.g. the peanut (*Arachis hypogaea*), store fat also. A steady flow of soluble nitrogenous compounds reaches the developing seeds from other parts of the plant. These materials are largely converted to protein but even the mature dry seed contains some soluble nitrogen; the proportion may be fairly high, Petrie (1908) recorded 28.5 per cent of the total seed nitrogen in *Acacia leptoclada* and 33.7 per cent in *A. pycnantha*. Dormant seeds contain amino-acids and amides (Portes, 1876; Kudryashova & Kolobkova, 1953). The absolute amount of non-protein nitrogen per seed increases even during the later stages of ripening in *Phaseolus vulgaris* (Pfenninger, 1909) and in *Vicia sativa* (Petrie, 1911a); it decreases in the bean (*Vicia faba*) (Emmerling, 1900) and in the pea (*Pisum sativum*) (Schulze & Winterstein, 1910; Bisson & Jones, 1932; McKee, Robertson, & Lee, 1955). In the soybean (*Glycine max*) protein nitrogen and non-protein nitrogen both increase linearly over the ripening period on a per seed basis; allantoic acid per seed increases steadily, the ureide being quantitatively more important than the amides in this species (Sosa-Bourdouil, Brunel, & Sosa, 1941). Considerable synthesis of protein occurs in seeds of *Lupinus albus* ripening in detached fruits (Vasiliev, 1908; Mothes, 1939), and in isolated immature pea seeds (Kertesz, 1930; Danielsson, 1952b). Zaleski (1911) showed that in isolated pea seeds the increase in protein nitrogen was roughly equivalent to the decrease in amide nitrogen plus that of compounds precipitated by phosphotungstic acid. These include

arginine, a major component of the soluble nitrogen in the pea seed (Schulze, 1911, Spragg, 1955) The immediate sources supplying nitrogen for protein synthesis in the developing per seed are thus probably arginine and amide, the latter is mainly glutamine (Spragg 1955) Numerous soluble nitrogenous compounds, including a wide range of amino acids, occur in immature pea seeds (Schulze & Winterstein, 1910, Hyde, 1953, Bisset, 1954, Spragg 1955, McKee, Nestel, & Robertson, 1955) The total soluble nitrogen per seed falls considerably in the early stages of ripening and then remains steady at a low level while protein nitrogen per seed increases at a linear rate The qualitative composition of the soluble nitrogen does not change greatly, most of the amino acids being present in small amounts in almost mature seeds

In the legumes the hull (carpel wall) acts as a temporary reservoir for nitrogenous and other substances in transit to seeds from other parts of the plant This is apparent in *Vicia faba* (Emmerling, 1900, Petrie, 1911a), *Phaseolus vulgaris* (Pfenninger, 1909, Schellenberg 1916) *Pisum sativum* (Bisson & Jones 1932, Hyde, 1954, McKee, Robertson, & Lee, 1955), and *Glycine max* (Sosa Bourdoul, Brunel, & Sosa, 1941) Most of the amino acids and amides found in immature seeds occur also in pea hulls, allantoin is an important constituent in this species (Schulze, 1911, Schellenberg, 1916) and allantoic acid in the hulls of soybean (*Glycine max*) (Sosa Bourdoul *et al*, 1941) Raacke (1957c) found that breakdown of the protein accumulated by pea hulls in the early stages of ripening led to peptides, which were translocated to the developing seeds Secondary synthesis of amides occurred in the hull, the amides also passing to the seeds In the hulls and also in the seed coats the protein is mainly, perhaps entirely, albumin Peptides accumulate in the seed coat (Raacke, 1957b) Protein nitrogen per hull rises in the early stages of ripening and falls later, most of the nitrogen left in the hull of the mature fruit is protein, whose persistence contrasts with the almost complete disappearance of starch In the early phases of ripening a substantial part of the protein in the hull may be in photo synthetically active chloroplasts Lubimenko (1910) investigated the composition of the gas contained in the hollow fruits of *Colutea arborescens* (Leguminosae) and found that in the light the carbon dioxide content decreased, with oxygen increasing at the same time The outer green parts of the hull appeared to assimilate carbon dioxide coming both from the external atmosphere and from respiration of developing seeds and the hull itself Calvert & Ferrande (1844) showed that the gas within these fruits had up to 3 per cent of carbon dioxide Photosynthesis



is significant in young fruits of pea and apple (Kursanov, 1934) and of tomato (Kursanov & Vartapetyan, 1956). In ripening seeds the insoluble materials protein and starch form an increasing proportion of the nitrogenous and carbohydrate reserves (Table 10).

TABLE 10

*Changes in proportions of soluble and insoluble nitrogenous compounds and carbohydrates in hulls and seeds of Pisum sativum.*  
(Calculated from data of McKee, Robertson & Lee, 1955.)

Days from flowering	Hulls		Seeds	
	Protein N as Per cent total N	Starch as Per cent (starch + soluble carbohydrate)	Protein N as Per cent total N	Starch as Per cent (starch + soluble carbohydrate)
14	56	—	40	—
18	55	—	50	—
20	53	25	57	19
23	64	16	61	27
26	61	17	87	43
29	59	12	84	63
32	59	8	86	73
35	69	3	90	81
40	87	3	93	81

Snellmann & Danielsson (1953) found peptides containing two to six amino-acid residues in immature pea seeds. The decrease in dialysable nitrogen and the increase in globulin nitrogen agreed well at successive stages of ripening, suggesting that peptides as well as amino-acids are intermediates in protein synthesis. This conclusion is supported also by the data of Raacke (1957a). In the early stages the loss of amino-nitrogen was too small to account for all the globulin nitrogen formed. This observation led to a suggested scheme of synthesis in which amino-groups were liberated during the formation of polypeptides from oligopeptides. Danielsson (1952b) used sedimentation analysis to study the synthesis of different types of protein in ripening pea seeds. Two globulins, legumin and vicilin, and an albumin fraction were synthesized at different rates, the proportion of vicilin decreasing in the later samples. Albumin was formed at a slow and steady rate throughout the ripening process. Raacke (1957a) found that very young pea seeds contained only albumin; vicilin appeared next and finally legumin.

The nitrogen/sulphur ratio in the protein of developing seeds of *Lupinus albus* remains steady in the early stages of ripening and then increases sharply (Mothes, 1939). A similar trend is shown in the data

of Emmerling (1900) for maturing seeds of *Vicia faba*. The changing ratio implies differential rates of synthesis for proteins rich and poor in sulphur containing amino acids. Byvshikh (1960) found that the proportion of dicarboxylic amino acids in the globulins of water melon seeds decreased during ripening with corresponding increases in arginine, histidine, lysine, proline and tryptophan.

Changes in the enzymatic activities of ripening seeds (Bach, Oparin & Walner 1927; Oparin & Dyachkov 1928) may reflect varying rates of synthesis of individual enzymatic proteins. Enzymatic activity, being sensitive to accelerators, inhibitors and other modifying factors, may not, however, be a good measure of the amount of enzyme protein present.

Special requirements are recorded for the synthesis of some enzymes. Zinc is essential for the synthesis of pyruvic carboxylase by *Rhizopus nigricans* (Foster & Demson 1950) and of phosphofructokinase, glycer aldehyde phosphate dehydrogenase and an enzyme involved in pentose metabolism by *Aspergillus niger* (Bertrand & de Wolf 1957, 1958b); it is not needed for invertase synthesis (Bertrand & de Wolf 1958a). Zinc deficiency greatly reduces the production of aldolase, the enzyme catalysing the reversible reaction between hexose diphosphate and triose phosphate, in oats (*Avena sativa*) and subterranean clover (*Trifolium subterraneum*) (Quinlan & Watson 1951). None of these enzymes is known to contain zinc. Zinc deficiency appears to reduce synthesis of the protein part of the enzyme molecule. Even with a zinc containing enzyme, carbonic anhydrase, zinc deficiency acts by reducing synthesis of enzymatic protein rather than through lack of zinc ions to activate an apoenzyme (Wood & Sibly 1952). Varying zinc requirements for the synthesis of different enzymes suggest that it is closely associated with the formation of some individual proteins though not necessarily with protein synthesis in general. This is consistent with the finding (Bertrand & de Wolf 1959, 1960) that it is essential for the synthesis of tyrosine and of tryptophan in *Aspergillus niger*. The synthesis in seeds and elsewhere of individual enzymatic and other proteins may thus be influenced by non-nitrogenous metabolites as well as by more immediate factors such as the availability of the appropriate amino acids.

### (iii) Protein synthesis in cereal grains

Kiesel (1924b) analysed rye grain (*Secale cereale*) at three stages of maturity, expressing his data in amounts per 100 ears of the substances

estimated. Protein nitrogen per ear increased continuously throughout the ripening period; non-protein nitrogen per ear was about the same in the first and last samples, but fell from 27 per cent to 13 per cent of the total nitrogen. Individual constituents found in the grain at various stages included adenine, arginine, aspartic acid, choline, guanidine, guanine, histidine, hypoxanthine, phenylalanine, putrescine, xanthine, and probably agmatine. In contrast to the array of purines in this list, no asparagine could be detected, though it was sought in samples of 4.5 kg in the early stages and of 6 kg later. Nedokuchayev (1897) also found very little asparagine in immature rye grain.

The amide content of ripening ears of wheat (*Triticum*) is also extremely low. Woodman & Engledow (1924) analysed wheat ears taken at intervals of a few days from 33 to 65 days after their emergence, the grain being fully mature in the last sample. Results were recorded as amounts in the grain of 100 ears. Total nitrogen on this basis increased steadily and rapidly for 54 days after emergence of the ears but much more slowly thereafter. The increase in non-protein nitrogen ceased at 47 days; during the next 7 days it decreased and appeared to contribute nitrogen for protein synthesis. Non-protein nitrogen as a percentage of total nitrogen fell from 32 in the first sample to 7 in the sample taken at 54 days. Amino and amide nitrogen were low throughout; about half the total soluble nitrogen was recorded as ammonia nitrogen in the later samples. The excess of ammonia over amide nitrogen is too great to be explained by inclusion of the amide nitrogen of glutamine in the figure for ammonia; hydrolysis of some labile non-amide constituent cannot, however, be excluded. Further work on the non-protein nitrogenous constituents in developing grain of wheat, rye, and other cereals should be of interest. Quantitative study of the numerous compounds reported by Kiesel (1924*b*) is desirable.

Kretovich & Yevstigneyeva (1949) found very little glutamine in ripening wheat ears. They placed cut wheat stems, carrying ears with grain at the milk-ripe stage, in solutions containing ammonium aspartate and ammonium glutamate. The solutions were rapidly taken up through the transpiration stream. Slight synthesis of asparagine occurred in ears supplied with water alone, and a little more with the ammonium salts. Addition of glucose to the nutrient solution reduced the synthesis of asparagine. No treatment induced any synthesis of glutamine. Koblet (1940) reported both asparagine and glutamine in the embryo of the developing wheat grain. He found that at the time of flowering the wheat plant already contained most of the nitrogen

required for seed formation, the carbohydrate laid down in the grain was in contrast largely synthesized during the ripening period. In corn (*Zea mays*) Hay, Larley & de Turk (1953) found that about 40 per cent of the nitrogen deposited in the grain was either absorbed from the soil after flowering or translocated from the roots which seem unlikely to be an important site for the storage of nitrogenous materials in this species. Reeves (1954) increased the protein content of wheat by urea sprays at flowering, spraying before flowering increased the yield but had less effect on protein content.

Woodman & Engledow (1924) estimated salt soluble, ethanol soluble, and alkali soluble proteins in wheat grain sampled on 9 occasions between 33 and 65 days after emergence of the ears, the final samples being mature. In the earliest sample salt soluble protein contained 74 per cent of the total protein nitrogen, seven days later its proportion had fallen in spite of an absolute increase in its amount to 48 per cent. The alkali soluble gluten increased rapidly over the first 14 days and remained thereafter essentially unchanged in absolute amount. The ethanol soluble gliadin increased steadily over the whole ripening period and contained 54 per cent of the protein nitrogen in the ripe grain. McCalla (1938) separated the proteins of developing wheat into fractions soluble in water, soluble in normal potassium iodide solution, and insoluble in normal potassium iodide. In the early stages of ripening the grain contained a labile water soluble protein subsequently converted to the water insoluble protein of the mature grain. The protein (glutelin) insoluble in a normal solution of potassium iodide was laid down early in the development of the grain, later accumulation of protein being as prolamins (soluble in normal potassium iodide). It is difficult to compare with certainty the results of Woodman & Engledow (1924) and of McCalla (1938) owing to the different solvents used to separate types of protein. The data of the two investigations are however, in general agreement on the plausible assumption that the gliadin and gluten of the former workers correspond respectively to the prolamins and glutelins of McCalla (1938). Seeds of *Pinus densiflora* and *P. thunbergii* contain mainly albumins in the early stages of development, glutelins predominate later, globulins also increasing to a lesser extent (Katsuta, 1959).

#### D Protein Synthesis in Vegetative Storage Organs

Some protein synthesis occurs in the cells of growing vegetative storage organs. The mature organs generally enter a dormant state in

which there is little protein synthesis and the ratio of soluble nitrogen to protein nitrogen is high. Rapid synthesis of protein takes place, however, when dormancy is broken and growth of new organs begins. Dormant storage organs such as tubers also often respond to wounding by a synthesis of protein associated with renewed growth at the cut surface.

Protein metabolism in onion bulbs was studied extensively about 1900 by a group of Russian workers. In the mature bulb a low proportion of the total nitrogen occurs in protein. Zaleski (1898) and Prianshnikov (1899) showed that during germination either in light or darkness a considerable part of the soluble nitrogenous material of the bulb was converted to protein. The main soluble precursors of protein were amino-acids, the asparagine content showing little change (Zaleski & Shatkin, 1913). Amino-acids rather than asparagine also appear to be the immediate precursors of protein in potato (Stuart & Appleman, 1935) and in disks of radish roots (*Raphanus sativus*) (Said & El Shishiny, 1944). A definite synthesis of protein at the expense of soluble nitrogenous constituents occurs before the start of germination. Zaleski (1901) found protein to contain 33 per cent of the total nitrogen in onions put into storage in the autumn (September). This proportion was unchanged in January, and during the next two months protein was synthesized until in February it contained 42 per cent and in March 53 per cent of the total nitrogen of the bulbs. Synthesis thus occurs even at the low temperatures of a cellar in Moscow during the winter, and is largely complete before any great rise in ambient temperature is likely. There is no synthesis in the autumn, when temperatures are comparatively high; at this time the bulbs, having completed their development, have just entered the dormant phase.

Wounding induces a large and rapid synthesis of protein in onion bulbs (Hettlinger, 1901; Zaleski, 1901). Zaleski (1901) observed increases in protein as a percentage of total nitrogen from 32 to 49, and in another experiment from 48 to 58, within four days after cutting bulbs into quarters. A further slight increase in the proportion of protein occurred in bulbs cut into numerous strips. Oxidative processes appeared to be involved, probably in the supply of energy for synthesis, as the protein content remained unchanged in strips held in an atmosphere of hydrogen. Smirnov (1903) found that in air wounding induced protein synthesis and increased respiration of cut onion bulbs; it had no effect on either process in an atmosphere of hydrogen. This confirmed

the results of Zaleski (1901) and supported the suggestion of a link between protein synthesis and respiration. The protein formed in cut tissue contained a higher proportion of nucleoprotein than in intact bulbs (Kovchov, 1902, 1903). Zaleski (1901) also recorded protein synthesis as a response to wounding in fleshy roots and tubers (*Apium graveolens*, *Beta vulgaris*, *Daucus carota*, *Dahlia variabilis*, and *Solanum tuberosum*). In these experiments as in the work with onion bulbs, stringent precautions were taken to avoid bacterial contamination.

Other work on protein synthesis in the tissues of fleshy storage organs has dealt mainly with the potato (*Solanum tuberosum*). Here also wounding induces a large and rapid increase in respiration rate (Richards, 1896). Potato tubers respond to a transfer from 0°C to 25°C by protein synthesis (Levitt, 1946); prolonged storage at 2°C, however, induces protein breakdown and after about 85 days the tubers lose their ability to synthesize protein and to form new tissue at a cut surface (Steward, Berry, Preston, & Ramamurti, 1943). The influence of external conditions on protein synthesis by disks of potato tuber is complex, but in general protein synthesis and respiration tend to be affected in the same direction (Steward, Stout, & Preston, 1940; Steward & Preston, 1941a, b). Protein synthesis is generally associated with increased respiration, as might be expected considering that it requires energy provided by respiration, and in most cases produces new cellular material whose integrity can only be maintained by respiration.

## D. BIOCHEMISTRY OF PROTEIN SYNTHESIS

### A. Proteolytic Enzymes in Plants

The most celebrated proteolytic enzyme of plant origin is undoubtedly papain from the latex of *Carica papaya* (papaya, pawpaw). The enzyme is produced commercially on a large scale as a tenderizer for meat. Tough meat wrapped in pawpaw leaves becomes tender, as is stated (Dujardin-Beaumetz & Égasse, 1889) to have been recorded about the middle of the eighteenth century by Griffith Hughes (*History of Barbados*) and Patrick Browne (*Natural History of Jamaica*); it is probably traditional knowledge in South America and the West Indies, where the plant is native. The latex, which dissolves the tapeworm *Ascaris*, is also an effective vermifuge; Vauquelin (1799) reported its use for this purpose in Réunion, a French colony in the Indian Ocean. Berger & Asenjo (1940) showed that *Ascaris* was

thoroughly digested by crystalline papain. Fresh pineapple juice, which contains the proteinase bromelin, also dissolves intestinal parasitic worms (Berger & Asenjo, 1939).

Papain was first studied by Wurtz & Bouehut (1879), who coined the name now current, and by Peckolt (1880) who used the less euphonious name papayotin. Both workers obtained preparations actively digesting animal proteins. Two distinct protein-splitting enzymes have been prepared in crystalline form from pawpaw latex, papain (Balls & Lineweaver, 1939) and chymopapain (Jansen & Balls, 1941). Similar enzymes are known from several other plants. Bouehut (1880) recorded proteolytic activity in the latex of the European fig (*Ficus carica*); Vines (1902) showed that such activity is retained in the dried fruit. Walti (1938) prepared the crystalline enzyme ficin and noted that in Central America the latex of several species of *Ficus* was used as a vermifuge. Carpenter & Lovelace (1943) obtained a crystalline proteinase (asclepain) from the root latex of *Asclepias speciosa*. Ellis & Lennox (1942) found a proteinase in the latex of *Euphorbia lathyris*. Other laticiferous species containing similar enzymes are *Hura crepitans* (Euphorbiaceae) (Jaffe, 1943a) and *Tabernaemontana grandiflora* (Apocynaceae) (Jaffe, 1943b). Proteinases also occur in fruits and leaves of non-laticiferous plants, e.g. bromelin in the pineapple (*Ananas comosus*) (Chittenden, 1894; Willstätter, Grassmann, & Ambros, 1926; Berger & Asenjo, 1939) and pinguinain in *Bromelia pinguin* (Asenjo & Capella de Fernandez, 1942). This species, like the pineapple, belongs to the family Bromeliaceae. Other plant proteinases include mexicain from latex in the leaves and fruit of *Pileus mexicanus* (Caricaceae) (Castañeda, Gavarrón, & Balcazar, 1942), solanain from the fruit of *Solanum elaeagnifolium* (Greenberg & Winnick, 1940), and actinidin from fruit of *Actinidia chinensis* (Arcus, 1959). Mexicain was crystallized by Castañeda-Agulló, Hernández, Loeza, & Salazar (1945). Crystalline proteinases have also been prepared from bacteria (Guntelberg & Ottesen, 1952) and moulds (Crewther & Lennox, 1950).

The presence of proteolytic enzymes in germinating seeds was established for a *Vicia* by Gorup-Besanez (1874b) and for *Lupinus hirsutus* by Green (1887). Buscalioni & Fermi (1898) and Vines (1903) detected such enzymes in various organs of numerous species widely scattered through the plant kingdom. Butkevich (1900, 1901) demonstrated the liberation of amino groups during autolysis of seedlings of *Lupinus*, *Ricinus*, and *Phaseolus*; he also obtained leucine and tyrosine by the action of crude enzyme preparations from seedlings on con-

glutin, the globulin of lupin seeds. The protein splitting enzymes of seedlings have received little study by exact methods. Blagoveshchenski (1924) and Blagoveshchenski & Melamed (1934) prepared seed globulins and crude proteolytic extracts from species belonging to several genera. Extracts and proteins were incubated in many different combinations, the degree of hydrolysis being always greatest when both substrate and enzyme came from the same species. In some combinations no hydrolysis occurred; in these cases the plants providing the enzyme and the substrate always came from different families. Differential rates of breakdown for separate protein fractions have been demonstrated by modern methods in germinating barley (Säverborn, Danielsson, & Svedberg, 1944) and peas (Danielsson, 1951).

Papain and similar enzymes, as stressed by Vines (1902) and Mendel & Blood (1910), are activated by hydrogen cyanide. Other reducing agents such as hydrogen sulphide, cysteine, and glutathione are also effective (Bersin & Logemann, 1933, Hellermann & Perkins, 1934; Purr, 1935). Winnick, Cone, & Greenberg (1944) found that a crystalline ficin required no activators if its oxidation was prevented; in less highly purified systems, and *in vivo*, activators may protect the enzyme from oxidation and from inhibiting heavy metals. It is possible that the active form of papain and similar enzymes has free sulphhydryl groups, and is inactivated by oxidation to a disulphide compound. This view has been supported by many workers, e.g. Bersin (1935), but is still not universally accepted. Some proteolytic enzymes, e.g. solanain, are not activated by hydrogen cyanide or hydrogen sulphide (Greenberg & Winnick, 1940). Comparison of the effect of activators on different enzymes is difficult unless, as is rarely possible, each is tested in identical conditions in relation to oxidoreduction potential and the presence of impurities. The sensitivity of proteolytic enzymes to activation and inhibition may be important for regulation of their activity within the cell.

Crystalline papain is a prolamin, being soluble in 70 per cent ethanol. Its molecular weight is 20,700 when prepared from dried latex, but about 27,000 when prepared from fresh latex. The molecule appears to consist of a single peptide chain. Most of the usual amino acids are present, except methionine. An unusual feature is the high content of tyrosine, which on a weight basis is the most abundant amino-acid in the molecule, followed by glutamic acid and aspartic acid (Kimmel & Smith, 1954, Smith, Kimmel, & Brown, 1954; Smith, Stockell, & Kimmel, 1954).



## B. Formation of Plasteins

Plastein is a general term for ill-defined insoluble products formed by proteolytic enzymes from concentrated protein hydrolysates. Danilevski (1886) and Mikhailov (1886) recorded such a reversal of the proteolytic action of pepsin. The condensation of peptides by proteolytic enzymes was confirmed by later workers, but the nature of the products and their relation to protein have caused much controversy. Lavrov (1907) showed that plasteins contained sulphur. Henriques & Gjaldbak (1911) synthesized plasteins in which few free amino groups could be detected by the formol titration method. Collier (1940), using crystalline papain, obtained from the digestion products of egg albumin a material with few free amino or carboxyl groups. Virtanen & Kerkkonen (1948) reported that pepsin formed peptides of molecular weight about 300. These clearly could contain only a few amino-acid residues, but were considered to be of cyclic structure as they showed few free amino groups. Such a structure could also be invoked to explain the paucity of amino groups observed by some earlier workers in products of unknown molecular weight. Later work from the same laboratory (Virtanen, Kerkkonen, Laaksonen, & Hakala, 1949; Virtanen, Kerkkonen, Hakala, & Laaksonen, 1950) led, however, to the conclusion that pepsin synthesized polypeptides containing on the average about 40 amino-acid residues and with molecular weights up to 10,000. These peptides were not formed from mixtures of amino-acids, or of dipeptides and tripeptides, the enzyme requiring more complex peptides as a substrate. Tauber (1951*a, b*) reported the synthesis of much larger molecules (molecular weights from 250,000 to 400,000) by chymotrypsin acting on peptides. Afanasyev & Talmud (1952) state that plastein is formed in peptone solution if pepsin is replaced by benzene, benzaldehyde, benzoic acid, toluene, or xylene. Horowitz & Haurowitz (1959) synthesized plasteins from small peptides with pepsin and chymotrypsin; they found that esters of various  $C^{14}$ -labelled amino-acids, but not the free amino-acids themselves, were incorporated into plastein and concluded that it was formed essentially by *transpeptidation* reactions.

Plastein formation shows the reversibility, in conditions involving no large change in free energy, of hydrolysis by some protein-splitting enzymes; it may not, however, be closely related to protein synthesis *in vivo*. There is other evidence that protein synthesis from peptides requires little energy. Butler (1946) made a rough calculation of the energy changes involved in this synthesis, and concluded that complete

oxidation of a glucose molecule provided sufficient energy to condense about 100 amino-acid residues to protein. Resynthesis of proteins from their hydrolysates by proteolytic enzymes at pressures of the order of 10,000 atmospheres was reported by Bresler (1947) and by Bresler & Glikina (1947). Bresler & Selezneva (1952) hydrolysed serum albumin by trypsin and chymotrypsin. The hydrolysate, containing peptides with an average of five amino-acid residues, was used for resynthesis at 6000 atmospheres in the presence of 20 per cent glucose to stabilize the enzymes. The product behaved in the ultracentrifuge very similarly to the original protein, but contained some material of different molecular weight. Bresler, Glikina, Selezneva, & Finogenov (1952) repeated this work with other proteins, and noted that the synthesis was a sudden rather than a gradual process. The synthesis was inhibited by mixed substrates. Bresler, Glikina, & Tongur (1951) hydrolysed insulin with chymotrypsin to inactive fragments of low molecular weight, and resynthesized it at pH 8.8 and 6,000 atmospheres to the biologically active hormone. These observations suggest that protein may in some circumstances be resynthesized from its hydrolysis products without a large input of energy, but other considerations indicate that in general protein synthesis follows a pathway different from the reversal of hydrolysis. Talwar & Macheboeuf (1954) were unable to repeat the observations of Bresler and his colleagues. Increased viscosity was noted, but no synthesis of peptide bonds could be established. The enzymes used became inactivated at high pressures.

### C. Synthesis of the Peptide Bond

Formation of the peptide bond is an endothermic process. The heat of formation of this bond varies considerably with the configuration of the reacting molecular species; it is generally estimated at 3,000 to 4,000 calories in the synthesis of simple amides and peptides, but may be less for peptide bonds formed in condensation of polypeptides (Borsook, 1953). The equilibria of the reactions catalysed by proteolytic enzymes are in aqueous solution far to the side of hydrolysis for peptides with even moderate solubility in water. Peptide synthesis by these enzymes requires that the peptides formed be removed from the reacting system, either by participation in some further reaction or by precipitation owing to low solubility.

Formation of peptide bonds by proteolytic enzymes was first demonstrated in a well defined system by Bergmann & Fraenkel-Conrat (1937), who synthesized substituted peptides precipitated below

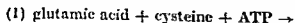
their equilibrium concentration. Papain acting on a concentrated solution of leucine anilide and benzoyl-leucine formed a peptide bond with production of benzoylleucyl-leucine anilide. Bergmann & Fruton (1938) obtained 65 per cent of the theoretical yield in condensing benzoyl-tyrosine and glycine anilide to benzoyl-tyrosyl-glycine anilide with chymotrypsin. The yield of the more soluble peptide formed from benzoyl-tyrosine and glycine amide was in similar conditions about 1 per cent (Fruton, Johnston, & Fried, 1951). Chymotrypsin requires neither free amino nor free carboxyl groups in substrates for hydrolysis. In synthetic reactions it acts on compounds containing combined amino-acid residues rather than on free amino-acids. Kaganova & Orekhovich (1954) found that it coupled the ethyl ester of tyrosine with amides, esters, or peptides of aspartic acid, glutamic acid, and leucine but not with the free amino-acids.

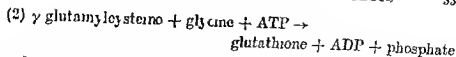
Some results with preparations from animal tissues suggest that *in vivo* protein breakdown requires energy or is tied to some energy-producing process. Simpson (1953) injected  $S^{35}$ -labelled methionine and  $C^{14}$ -labelled leucine into intact rats, and followed the breakdown in liver slices of proteins incorporating these radioactive amino-acids. Protein breakdown, as measured by the appearance of labelled methionine and leucine, was inhibited in intact cells by inhibitors of respiration and of protein synthesis; neither affected breakdown in disrupted cells. Steinberg, Vaughan, & Anfinsen (1956) reported similar results and found that *o*- and *p*-fluorophenylalanine inhibited both synthesis and breakdown of protein.

#### D. Phosphorylation and the Synthesis of Peptide Bonds

The stimulation by phosphate of protein synthesis in disks of potato tuber tissue led Steward & Preston (1940, 1941b) to suggest that phosphorylated nitrogenous compounds were involved in the formation of protein. Lipmann (1941) made similar suggestions by analogy with the rôle of phosphorylations in other biosynthetic processes. Black & Gray (1953) found in yeast an enzyme forming aspartyl phosphate from aspartic acid and adenosine triphosphate.

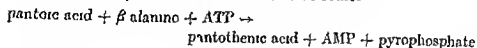
The tripeptide glutathione ( $\gamma$ -glutamylcysteinylglycine) is synthesized in liver and yeast (Bloch & Anker, 1947; Bloch, 1949; Snoke, 1953; Snoke & Bloch 1952, 1955; Mandeles & Bloch, 1955) by the reactions:





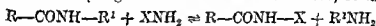
Phosphorylated enzymes probably take part in these reactions as in the synthesis of glutamine. Enzyme systems catalysing glutathione synthesis occur in higher plants (Webster, 1953 *b c* Webster & Varner, 1954a, b, 1955a). Virtanen & Ettala (1958) recorded another  $\gamma$  glutamyltripeptide ( $\gamma$  glutamyl- $\gamma$ -glutamic acid) in *Juncus conglomeratus*, *J. effusus*, and *J. filiformis*.

The synthesis of pantothenic acid in bacteria (Maas 1952, Ginoza & Alternbern, 1955) follows a somewhat similar course



## E Transamidation and Transpeptidation

Proteases as well as hydrolysing peptide bonds also catalyse transfer reactions (Bergmann & Fraenkel Conrat 1937) of the type



It is probable that an enzyme peptide compound is formed which reacts either with water, leading to hydrolysis or with an amine which accepts a complex group transferred from the peptide molecule. Johnston, Mycek, & Fruton (1950) showed that papain catalysed exchange of the amide group of benzoylglycylamide with  $\text{N}^{15}$  labelled ammonia and with hydroxylamine. Fruton, Johnston & Fried (1951) obtained transfer of several more complex groups by papain and by ficin.

Stumpf, Loomis & Michelson (1951) found in higher plants a widely distributed  $\gamma$  glutamyl transferase catalysing transfer of  $\gamma$  glutamyl groups from glutamine to hydroxylamine or to  $\text{N}^{15}$  labelled ammonia. In contrast to the somewhat similar transfer reaction catalysed by papain, hydrolysis did not accompany the transfer. The enzyme was highly specific for glutamine. Transpeptidases catalysing exchange reactions between peptides and free amino acids occur in plant and animal tissues (Hanes, Hird & Isherwood 1952, Kaganova & Orekhovich, 1953). Cathepsin catalyses the condensation of two molecules of alanylphenylalanine amide to a tetrapeptide, which in turn combines with the original dipeptide to form a hexapeptide, one molecule of ammonia being eliminated at each condensation (Fig. 60) (Fruton, Hearn, Ingram, Wiggans & Winitz 1953). Medvedyev & Shen (1959) supplied  $\text{C}^{14}$  labelled peptides to detached leaves of *Phaseolus vulgaris*.

and *Thermopsis officinalis* (Leguminosae). Radioactive carbon appeared in the leaf proteins, suggesting that the peptides were used in their synthesis.

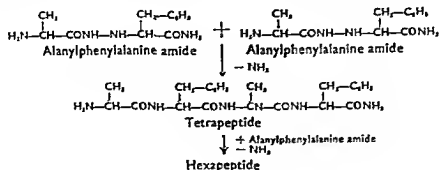
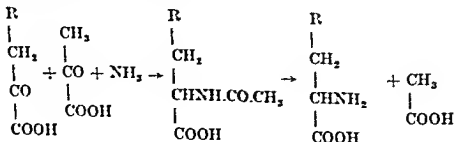


FIG. 60.

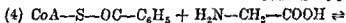
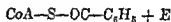
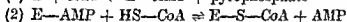
## F. Activation of Amino-acids

Activation is an ambiguous term used with more than one meaning in the chemical and biochemical literature. In studies of chemical kinetics an activated molecule is one which has acquired an energy content higher than the average, enabling it to enter a reaction with a definite threshold energy level. In biochemistry an activated molecule is usually an intermediate compound more reactive than its precursors or the final products of the reaction sequence. These labile intermediates, being difficult to isolate, are rarely recognized as reactants in early studies of a reaction sequence, though chemical considerations may suggest their existence. Known or postulated reactive derivatives are often referred to as 'activated', though the kinetically activated molecular species taking part in the key reactions are more likely to be enzyme-substrate complexes. The word 'activation' thus has distinct biochemical and kinetic meanings which should not be confused with one another.

Knoop (1910) and du Vigneaud & Irish (1938) suggested that acetyl derivatives are intermediates in the synthesis of amino-acids and peptides, as in the sequence:



Bloch & Borek (1946) obtained deuterium labelled acetyl amino acids on incubating liver slices with labelled acetic acid and leucine, phenyl alanine, and phenylaminobutyric acid. The acetyl amino acid corresponding to the last named amino acid was not further metabolized and accumulated much more than the acetyl leucine and acetyl phenylalanine. Mutant strains of *Escherichia coli* that do not synthesize tyrosine and phenylalanine are however, unable to use their acetyl derivatives (Simmonds Tatum & Fruton 1947). An enzymatic acetylation of glycine precedes the formation of hippuric acid from glycine and benzoic acid in preparations from animal tissues. Both adenosine triphosphate and co enzyme A are involved, the suggested sequence of reactions is (Chantrenne, 1951, Schachter & Taggart, 1954)



(E = enzyme (glycine N acylase), ATP = adenosinetriphosphate)

Enzyme catalysed reactions forming a high energy bond between adenosine monophosphate and the carboxyl groups of various amino acids occur in preparations from animal tissues and micro organisms (Hoagland 1955, de Moss & Novelli, 1955, Hoagland Zamecnik, & Stephenson 1957, Cole, Coote, & Work, 1957, Nisemann, Bergmann, & Berg, 1957, Bernlohr & Webster, 1958). There is evidence (Webster, 1957a, b, 1959) for the occurrence of similar reactions in plant materials. The activating enzymes generally occur in the liquid remaining after removal of intracellular particles, some workers (e.g. Webster, 1957a, Weiss, Acs, & Lipmann, 1958) however, reported activation in particles. Work on enzymes catalysing the formation of amino acid adenylates has attracted much attention owing to their probable connexion with protein synthesis, and they are being actively studied in several laboratories. Some authors hold that a separate enzyme activates each of the amino acids built into the protein molecule, but this is not satisfactorily established. Reports on the subject are somewhat contradictory, clarification by further work is needed and may confidently be expected in view of the intense activity in this field. One possible source of confusion is the exchange of free tryptophan with its adenylate in the presence of the tryptophan activating enzyme (Karasch, Castel

franco, Krishnaswamy, & Meister, 1958). The occurrence in some tissues of other compounds between amino-acids and nucleotides may also complicate the study of amino-acid adenylates.

In silk-forming glands of the silkworm, activation of the carboxyl groups of amino-acids shows no correlation with their incorporation into the silk protein (Heller, Szafranski, & Sulkowski, 1959). Tryptophan and tyrosine showed the highest rate of activation, though neither was an important constituent of the protein synthesized. Glycine, a major protein constituent, was not activated. No transacylation to glycine was detected; its mode of incorporation thus remains doubtful. In bacterial (Beljanski & Ochoa, 1958) and animal (Cohn, 1959) systems there is evidence for the incorporation of amino-acids into protein in the absence of activating enzymes. There may therefore be pathways of protein synthesis not involving amino-acid activation by the mechanisms now known.

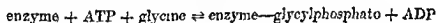
The simultaneous presence of all the amino-acids (or their active derivatives) occurring in a protein may be essential for its synthesis. Monod, Pappenheimer, & Cohen-Bazire (1952) showed that, in eleven mutants of *Escherichia coli* each requiring an extraneous source of a particular amino-acid, cell protein and the adaptive enzyme  $\beta$ -galactosidase were not synthesized in the absence of the essential amino-acid.

Uridine nucleotides combined with peptides accumulate in cells of *Staphylococcus aureus* treated with penicillin. The cell-walls of this bacterium contain a substance yielding on hydrolysis glutamic acid, alanine, and an amino-sugar. Transglycosidations involving uridine diphosphate nucleotides may take part in the synthesis of this cell-wall material. The nucleotide-peptide compound observed in penicillin-treated cells is probably an intermediate accumulating when its further metabolism is blocked by the antibiotic (Park, 1952; Park & Strominger, 1957). Synthesis of these cell-wall compounds involves an enzyme-AMP-D-alanine intermediate in *Lactobacillus arabinosus* (Baddiley & Neuhaus, 1959).

Adenylamino-acid anhydrides have been chemically synthesized (de Moss, Genuth, & Novelli, 1956; Karasek *et al.*, 1958); the latter workers also isolated adenyl tryptophan from the products formed by the tryptophan-activating enzyme acting on  $C^{14}$ -labelled tryptophan and adenosine triphosphate. The mixed anhydrides are highly reactive and indeed unstable compounds, reacting so rapidly with water that in neutral solution their half-lives are measured in minutes. This reactivity is in agreement with the behaviour of mixed anhydrides of amino-acids

and free or substituted phosphoric acids (Chantrenne, 1950, Bentler & Netter, 1953, Katchalsky & Paecht 1954) Labelled adeny amino acids transfer their amino acid portions to protein non enzymatically (Castelfranco, Moldave, & Meister, 1958) The synthetic mixed amino acid adenylic acid anhydrides also react much more rapidly with hydroxylamine than the enzymatic products in a reaction mixture It is therefore supposed (Hoagland, 1955, Davie Koningsberger, & Lipmann, 1956) that the latter remain firmly bound to the enzyme molecule on which they are formed This implies that in the complex of enzyme and mixed anhydride the acyl group of the amino acid is protected in some way against reactions in which it would normally participate in aqueous solution but is available for further enzymatic synthetic reactions

Cormier, Stulberg & Novelli (1959) obtained from *Photobacterium fischeri* an enzyme activating the carboxyl group of glycine Unlike the enzymes already mentioned it did not catalyse an exchange reaction between adenosine triphosphate and inorganic pyrophosphate either in the presence or the absence of glycine Studies with  $O^{18}$  labelled glycine suggested the following course for the activation



Amino acyl compounds of thioesters provide another type of reactive amino acid derivative Wieland & Schafer (1951, 1952) obtained such derivatives by the reaction of amino acid hydrochlorides with thiophenol Amino acyl derivatives of aliphatic mercaptans could not be obtained directly, but were synthesized by an acyl transfer reaction with derivatives of thiophenol These reactions transferred acyl groups to amino groups in physiological conditions, but were very slow Wieland, Bokelmann Bauer, Lang, & Lau (1953) found that the reaction was greatly accelerated with compounds e.g. cysteine and cysteamine, which had sulphhydryl and amino groups in the same molecule and in sterically satisfactory positions relative to one another In such cases acyl groups migrated rapidly from the sulphur atom to the amino group Similar rearrangements occur in S acyl peptides Wieland, Lang & Laebisch (1955) studied the rearrangements taking place on neutralization of S valyl N alanyl glycyl cysteamine This compound yielded three stable peptides with different arrangements of the four amino acid residues contained in the original peptide S acyl compounds of amino acids may thus play some part in protein biosynthesis through thiol linkages comparable to those formed by co-enzyme



A, but this remains to be established. It may be relevant in this connexion that 2-mercaptoethylamine increases the binding of labelled leucine to soluble ribonucleic acid, apparently by a process independent of amino-acid adenylates (Rendi & Hultin, 1959).

The main immediate interest of this work, and of similar insertions of amino-acid residues into existing peptides with other acyl-amino-acids (Brenner, Zimmermann, Wehrmüller, Quitt, & Photaki, 1955), lies in the entry of individual amino-acids into peptide chains without requiring their complete synthesis from the amino-acid level. This observation emphasizes the need to distinguish between incorporation of exogenous amino-acids and complete protein synthesis. Other workers (e.g. Castelfranco, Moldave, & Meister, 1958; Zioudrou, Fujii, & Fruton, 1958) have shown that amino-acid adenylates are incorporated into protein molecules by both enzymatic and non-enzymatic reactions. Sarkar, Clarke, & Waelsch (1957) and Clarke, Mycek, Neidle, & Waelsch (1959) showed that an enzyme system from mammalian tissues catalysed the incorporation into many proteins (though not into all that were tested) of a wide range of amines not known as normal constituents of protein. Among the amines incorporated in this way were alanine amide, cadaverine, glycine amide, ethanolamine, methylamine, phenylethylamine, putrescine, and spermine. Lysine was also incorporated, but none of the monoaminomonocarboxylic acids tested. The reaction required no extraneous source of energy. The amines were incorporated as such, cadaverine taken up by a protein being recovered from its acid hydrolysate. The amines may replace amide groups in the protein; ammonia was liberated during the reaction in amounts proportional to the uptake of amine.

Amino-acids not occurring naturally can be incorporated into protein. These include ethionine (an analogue of methionine) in *Tetrahymena pyriformis* (Gross & Tarver, 1956), azatryptophan in *Escherichia coli* (Pardee, Shore, & Prestidge, 1956) and *p*-fluorophenylalanine in the same organism (Munier & Cohen, 1956). Labelled norleucine supplied to cows is incorporated into the casein of their milk (Black & Kleiber, 1955). Methionine appears to be completely replaceable by its selenium analogue in *E. coli* (Cowie & Cohen, 1957). Protein-synthesizing mechanisms are thus far from completely specific when confronted with amino-acids outside their normal range. Within that range they may operate with greater precision.

## G. Nucleic Acids and Protein Synthesis

Caspersson (1941) and Brachet (1942) pointed out that the ribonucleic acid content of cells was closely correlated with their ability to synthesize protein. These authors and their co-workers showed in a wide range of animal tissues that cells actively synthesizing protein contained much more ribonucleic acid than cells of comparable origin which formed little protein, even if the latter were physiologically very active in other ways. A good example is provided by the silk-forming gland of the silk-worm; its only known function is the synthesis of silk fibroin (a protein) and it is very rich in ribonucleic acid (Brachet, 1942; Denucé, 1952); synthesis of fibroin, being inhibited by ribonuclease, appears to depend on intact ribonucleic acid (Takeyama, Ito, & Miura, 1958). In endocrine glands stimulated to produce protein hormones (Desclin, 1940; Herlant, 1943; Abolnš, 1952) or in gonads stimulated to produce reproductive cells (Schrader & Leuchtenberger, 1950, Rabinovitch, Junqueira, & Rothschild, 1951) there is a close connexion between protein synthesis and ribonucleic acid content. Fewer demonstrations of this relationship are available for plants, but it has been reported in germinating seedlings (*Vigna sesquipedalis*: Oota & Osawa, 1954; *Pisum sativum*: Webster, 1957b) and in the large unicellular alga *Acetabularia mediterranea* (Stich, 1951). Autoradiography shows in plant and animal tissues, a close topographical correlation between ribonucleic acid content and incorporation of  $C^{14}$ -labelled amino-acids (Ficq, 1955a, b; Brachet & Ficq, 1956). Penicillinase synthesis is induced by a nucleic acid (Kramer & Stranb, 1956).

Caldwell, Mackor, & Hinshelwood (1950) studied the synthesis of protein by bacterial cultures in the logarithmic phase of growth. Protein synthesis varied widely with environmental factors such as the nature and amount of the nitrogen supply or the presence of inhibitors, and with the type of organism; it was closely correlated with the ribonucleic acid content of the cultures. Bonnet & Gayet (1950) cited evidence that the ribonucleic acid of intracellular granules in micro-organisms was involved in protein synthesis. Gale & Folkes (1953a, c, d) also reported a close correlation between ribonucleic acid content and rate of protein synthesis in cultures of *Staphylococcus aureus* grown in a wide range of conditions and therefore forming protein at very varied rates. The antibiotics aureomycin, chloramphenicol (chloromycetin), and terramycin in bactericidal concentrations were, however, found to inhibit protein synthesis but to stimulate

the synthesis of nucleic acid (Gale & Folkes, 1953b). In *Escherichia coli* chloramphenicol and the structurally unrelated antibiotic erythromycin had very similar effects, both stopping protein synthesis without inhibiting formation of nucleic acid (Brock & Brock, 1959).

*E. coli* treated with chloramphenicol forms large amounts of ribonucleic acid. In cells subsequently transferred to media free of the antibiotic most of this material is excreted before growth, multiplication, and protein synthesis are resumed (Hahn, Schaechter, Ceglowski, Hopps, & Ciak, 1957). The authors suggest that the excreted material is a normal ribonucleic acid formed in excess of the amount required by cells that cannot synthesize protein. It may, however, be abnormal material ineffective in protein synthesis. Ben-Ishai (1957) and Horiuchi, Horiuchi, & Mizuno (1959) reported results suggesting that in *E. coli* protein synthesis requires a concurrent synthesis of ribonucleic acid, pre-formed ribonucleic acid being ineffective. A similar situation might explain the observation (Webster & Johnson, 1955) that in preparations from roots of pea seedlings protein synthesis was stimulated more by mixtures of purines, pyrimidines, nucleotides, and nucleosides than by added ribonucleic acid.

The antifungal polyene amphotericin B inhibits the synthesis of both protein and ribonucleic acid in the yeast *Candida albicans* (Drouhet, Hirth, & Lebeurier, 1958; Hirth, Lebeurier, & Drouhet, 1959a). It appears that this substance, which inhibits also the synthesis of carbohydrate reserve materials, acts by accelerating the conversion of adenosine triphosphate to adenosine diphosphate; it may activate adenosine triphosphatase (Hirth, Lebeurier, & Drouhet, 1959b). The relation between the syntheses of protein and nucleic acid seems not to be reciprocal; protein synthesis requires the presence, and perhaps the concurrent synthesis of nucleic acid, but the latter can be synthesized in conditions preventing protein synthesis. Some reports (Mitchell, 1950; Wisseman, Smadel, Hahn, & Hopps, 1954) suggest that protein synthesis in bacteria may not always be completely inhibited by chloramphenicol. There is, however, general agreement that this antibiotic affects the formation of protein much more strongly than that of nucleic acids.

Gale & Folkes (1954a, b; 1955) studied the effect of ribonuclease on protoplasts of *Staphylococcus aureus* disrupted by ultrasonic vibrations. Treated cells still showed net protein synthesis, and formed the adaptive enzyme  $\beta$ -galactosidase. Removal of ribonucleic acid with ribonuclease inhibited protein synthesis; the inhibition was reversible by addition

of ribonucleic acid or of a mixture of purines and pyrimidines from which it was synthesized in the cells. Addition of deoxyribonucleic acid also favoured protein formation. This effect was considered to be indirect, deoxyribonucleic acid acting as an organizer for the formation of specific ribonucleic acids involved in protein synthesis. Lester (1953) and Beljanski (1954) obtained similar results with bacteria lysed with lysozyme and then treated with ribonuclease. The lysed bacteria on treatment with ribonuclease lost almost completely their capacity to incorporate labelled amino acids into protein, this inhibition could not be attributed to a non specific effect on energy producing reactions as respiration was unaffected.

Ribonuclease, a protein of molecular weight over 12 000, appears somewhat surprisingly to enter intact roots. Kaufmann & Das (1954, 1955) found various mitotic anomalies in cells of roots of several species placed in a dilute solution of ribonuclease. Brachet (1954) treated intact onion roots with a solution of crystalline ribonuclease. Within one hour from the start of treatment incorporation of  $C^{14}$  labelled glycine and phenylalanine into the root proteins fell to 50 per cent of the initial rate, after three hours it was 10 per cent of the initial rate. The enzyme attacked soluble ribonucleic acid, its inhibition of protein synthesis was reversed by yeast ribonucleic acid (Brachet & Six 1950). Ribonuclease inactivated by gentle oxidation had no effect on the incorporation of amino acids. As in the bacteria studied by Beljanski (1954) ribonuclease had very little effect on the respiration of treated roots. Their rate of oxygen uptake was unaltered, but inorganic phosphate decreased and adenosine triphosphate increased (Brachet 1955a, 1956) after treatment with the enzyme. Brachet (1955b) varied the ribonucleic acid content of living amoebae widely by treatment with ribonuclease and found that incorporation into protein of  $C^{14}$  labelled phenylalanine varied directly with the ribonucleic acid content.

Work with plant viruses also supports the theory that ribonucleic acid is involved in the synthesis of protein. All plant viruses examined as crystals contain substantial amounts of ribonucleic acid which represents 10 to 40 per cent of their dry weight. Proteins closely resembling those of active viruses but free from ribonucleic acid have been isolated from infected plants. Such proteins are not infective and so fail to induce the synthesis of virus protein in a susceptible host plant (Markham & Smith 1949, Jeener 1954). This suggests that the ribonucleic acid component controls in some way the synthesis of virus protein. The multiplication of tobacco mosaic virus is inhibited

(Commoner & Mercer, 1952) by thiouracil, an analogue of uracil, one of the pyrimidine components of ribonucleic acid.  $S^{35}$ -labelled thiouracil supplied to infected tobacco leaves is incorporated into virus ribonucleic acid (Jeener & Rosseels, 1953; Matthews, 1956). The abnormal ribonucleic acid so formed is non-infective and therefore does not induce synthesis of virus protein. 8-Azaguanine, an analogue of guanine, a purine component of ribonucleic acid, also inhibits virus multiplication in this way (Matthews, 1951, 1953, 1954). Thiouracil is incorporated into ribonucleic acid in bacteria also; Hamers & Hamers-Casterman (1959) found that in *Bacterium megatherium* it replaced 20 per cent of the uracil. Bacteria containing this altered ribonucleic acid produced a protein resembling the  $\beta$ -galactosidase of normal cells but showing little or no enzymatic activity. The authors suggested that this protein was an altered enzyme formed under the influence of the thiouracil-containing ribonucleic acid. Creaser (1955) found that 8-azaguanine inhibited the substrate-induced synthesis of  $\beta$ -galactosidase by *Staphylococcus aureus*, the inhibition being reversible by guanine, hypoxanthine, or xanthine. He suggested that incorporation of 8-azaguanine produced an abnormal ribonucleic acid ineffective in protein synthesis. In *Bacillus cereus* up to 40 per cent of the guanine in ribonucleic acid can be replaced by 8-azaguanine (Smith & Matthews, 1957). The ribonucleic acid so formed is more acid-labile than the normal material of this species. Protein synthesis is inhibited within ten minutes after 8-azaguanine is added to the culture (Chantrenne & Devreux, 1958). 5-Fluorouracil can replace almost half the uracil of ribonucleic acid in *Escherichia coli* (Horowitz & Chargaff, 1959). Its incorporation into bacterial ribonucleic acid changes the amino-acid composition of protein formed subsequently (Naono & Gros, 1960).

Separation of the protein and ribonucleic acid of a virus and its resynthesis from these components were reported by Fraenkel-Conrat & Williams (1955) and Lippincott & Commoner (1956). This work was followed by the demonstration (Gierer & Schramm, 1956; Fraenkel-Conrat, Singer, & Williams, 1956) that the ribonucleic acid component of tobacco mosaic virus retained, independently of the protein portion, some infectivity, which was destroyed by digestion with ribonuclease. Synthesis of Semliki Forest virus (Cheng, 1958), of an influenza virus (Portocala, Boeru, & Samuel, 1959) and of a polyhedral insect virus (Krieg, 1959) is also induced by their ribonucleic acid components. Reconstitution of an infective virus by combination of protein and ribonucleic acid from two different strains of tobacco mosaic virus is

reported (Fraenkel-Conrat, 1956), the protein formed by multiplication of the 'hybrid' virus being that associated with the strain which supplied the ribonucleic acid component.

Interpretation of some of these data on the synthesis of tobacco mosaic virus is complicated by the low infectivity retained by the isolated ribonucleic acid. Even this slight infectivity is rapidly lost. It is therefore possible that the results considered to imply resynthesis of the virus indicate rather a stabilization by the protein of activity that would in its absence disappear before testing. Complete restoration of the original activity of a dissociated virus seems not to have been achieved as yet. Rod-shaped particles closely resembling those of tobacco mosaic virus are formed by combination of the virus protein with a wide variety of nucleic acids and even synthetic polymers of single nucleotides such as adenylic acid and uridylic acid (Hart & Smith, 1956). These particles, however, are not infective. Tobacco mosaic virus can lose its infectivity without any obvious change in the size or shape of the macromolecule (Gavrilova & Spirin, 1959). In spite of all these uncertainties it is clear that the ribonucleic acid plays a major part in determining the protein synthesis necessary for virus multiplication. It is, moreover, probable that a specific ribonucleic acid induces synthesis of the virus protein. There are also reports suggesting that in bacteria ribonucleic acid taken from strains producing particular enzymes can induce their formation in strains normally lacking them. This has been reported for gluconokinase in *Escherichia coli* (Reiner & Goodman, 1955), mannitol phosphatodehydrogenase in *Pneumococcus* (Marmur & Hotchkiss, 1955), penicillinase in *Bacillus cereus*, and  $\beta$ -galactosidase in *Bacterium megatherium* (Hunter & Butler, 1956).

Kessler (1956) found that spraying leafy branches of intact plants with a solution containing 50 p.p.m. of uracil increased the synthesis of protein and of ribonucleic acid in olive (*Olea europaea*) and grape (*Vitis vinifera*). Sprays containing methyltryptophan inhibited protein synthesis, presumably through interference with incorporation of tryptophan, but had no effect on the synthesis of ribonucleic acid. Thiouracil, an antagonist of uracil, inhibited the synthesis of both ribonucleic acid and protein, suggesting that in higher plants also synthesis of protein is closely associated with that of ribonucleic acid.

## II. The Site of Protein Synthesis in the Cell

Casparson (1941), Casparson & Thorell (1941), and Bracket (1942) stressed the association of protein synthesis with nucleic acids.

Caspersson (1950) suggested the nucleus as the main site of protein synthesis in the cell. Later work has continued to emphasize the powerful influence exercised by nucleic acids on protein synthesis, which is now known to occur both in the nucleus and the cytoplasm.

Numerous observations on animal material showed that nuclei, both within the cell and isolated from it, can synthesize protein; the nucleolus is particularly active in this respect (Daly & Mirsky, 1952; Smellie, McIndoe, & Davidson, 1953; Fieq, 1955*a, b*). In many tissues, however, protein synthesis in the cytoplasm seems to exceed that in the nucleus. Substantial synthesis of protein is possible in cells without a nucleus. Reticulocytes, enucleate cells developing into the red corpuscles of the blood, incorporate labelled amino-acids into protein and form specific proteins such as haemoglobin and several enzymes (London, Shemin, & Rittenberg, 1950; Holloway & Ripley, 1952; Koritz & Chantrenne, 1954; Rabinovitz & Olson, 1959). The large unicellular alga *Acetabularia mediterranea* provided very interesting data (Brachet & Chantrenne, 1951; Brachet, Chantrenne, & Vanderhaeghe, 1955) in this connexion. It was divided into two portions, one retaining the nucleus. In favourable conditions the enucleate portion regenerated rapidly, synthesizing large amounts of protein. The initial rate of synthesis even exceeded that of the nucleate portion. Protein synthesis, as measured by the incorporation of labelled glycine and (in the light) of labelled carbon dioxide into protein persisted for about two weeks after removal of the nucleus. Carbon from carbon dioxide was incorporated mainly into chloroplast proteins; labelled glycine appeared mainly in the microsome fraction of the cells. This work was confirmed and extended by Richter (1959) who found in nucleated growing cells of *Acetabularia* a constant ratio between ribonucleic acid and soluble cytoplasmic protein, both being synthesized steadily. Enucleate portions ceased to form ribonucleic acid, whose amount remained constant, but the content of soluble cytoplasmic protein increased for 21 days.

Loss of the nucleus, though without obvious immediate effect, finally prevented further growth, protein synthesis ceasing after two or at the most three weeks. Possibly the supply of some material, produced by the nucleus and necessary for growth, is exhausted in the enucleate portion of the alga.

There is, as mentioned earlier, much evidence that protein is synthesized in the chloroplasts. Mitochondria also synthesize protein (Webster, 1954; Bates, Craddock, & Simpson, 1958); its synthesis is

correlated with the rate of phosphate turnover in ribonucleic acid (Khesin, 1951) Ability to form protein thus appears in several sub cellular structures Plaut & Rustad (1959) found that in *Amoeba proteus* ribonucleic acid is synthesized in the cytoplasm as well as in the nucleus

Recent work stresses the importance of the microsomes in protein synthesis These intracellular particles are roughly spherical, with diameters from 200 to 300 Å (0.02 to 0.03  $\mu$ ), they may not be entirely homogeneous even in a single tissue They are considerably smaller than mitochondria, from which they are distinguished also by a high content of ribonucleic acid The correlation between ribonucleic acid content and protein forming activity appears to hold within the cell too Several workers found that labelled amino acids supplied to animals accumulated in the microsomes, especially in the liver (Borsook, Deasy, Haagen Smit, Keighley, & Lowy, 1950, Hultin, 1950, Lee, Anderson, Miller, & Williams, 1951) Microsomes take part in protein synthesis in other tissues of animals (Allfrey, Daly, & Mirsky, 1953) and plants (Oota & Osawa, 1954, Webster & Johnson, 1955, Cosentino, 1956)

Protein synthesis in the microsomes is coupled to an energy providing phosphorylation system (Siekevitz, 1952) The microsomes are poor in enzymes and their activity *in vivo* is probably linked to respiratory activity of the mitochondria *In vitro* the presence of mitochondria is not essential if some other system is provided to generate adenosine triphosphate (Zamecnik & Keller, 1954) These authors also concluded that incorporation of labelled amino acids into the proteins of rat liver microsomes required the presence of a soluble, heat labile, non dialysable fraction Hoagland, Zamecnik, & Stephenson (1957) demonstrated that soluble ribonucleic acid not attached to microsomes, is involved in protein synthesis Labelled amino acids incubated with adenosine triphosphate and soluble liver proteins precipitated at pH 5 (the 'pH 5 fraction' containing amino acid activating enzymes) were bound to soluble ribonucleic acid, from which they were transferred to the ribonucleic acid of the microsomes Similar results have been reported for other animal preparations by several workers, e.g. Weiss, Acs, & Lipmann (1958), and for micro organisms (Berg & Ofengand, 1958, Mager & Lipmann 1958) Individual amino acids appear to be bound independently and in definite amounts to the soluble ribonucleic acid (Hoagland, Stephenson, Scott, Hecht, & Zamecnik, 1958, Berg & Ofengand, 1958) Specificity of binding is sufficiently marked for glutamic acid and glutamine to be taken up independently (Traser, Shimizu, & Gutfreund 1959) Smith Cordes, & Schweet (1959)



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separated soluble ribonucleic acid from liver into fractions specifically incorporating isoleucine, lysine, threonine, and tyrosine, as activated compounds bound to an enzyme molecule. In disrupted cells of *Staphylococcus aureus* (Gale & Folkes, 1955) specific dinucleotides and trinucleotides promoted the incorporation of individual amino-acids (aspartic acid, glutamic acid, leucine). The amino-acids attached to soluble ribonucleic acid seem not to be linked in peptides. Nucleotide-peptide compounds are, however, reported from animal, fungal, and bacterial sources (Dirheimer, Weil, & Ebel, 1958) and from yeast (Koningsherger, van der Grinten, & Overbeek, 1957). The peptides are probably joined through their carboxyl groups to the nucleotides. The Dutch authors suggest that these compounds represent a stage in protein synthesis; their data are consistent with this conclusion but other interpretations seem possible. Harris & Davies (1959) isolated from yeast a nucleotide-peptide characterized as uridine-5'-phosphate combined with a tetrapeptide containing two molecules of alanine and two of arginine.

Busch, Weill, Ledig, & Mandel (1958) studied the effect of protein deficiency on the biosynthesis of ribonucleic acid in the liver cell-sap of intact rats. Prolonged protein deficiency led to a reduction in ribonucleic acid. Two fractions of ribonucleic acid were distinguished, the metabolically more active being also more stable in deficient animals. The synthesis of ribonucleic acid and of protein were both inhibited, as might be expected from the associations between these substances established in other work, and from the fact that certain amino-acids are precursors both of protein and of nucleic acid. Deficiency of amino-acids also suppressed the formation of ribonucleic acid in bacteria (Gale & Folkes, 1953b; Borek, Ryan, & Rockenbach, 1955). Nucleoside polyphosphates accumulated, but disappeared when amino-acids were supplied and protein synthesis began. It was suggested that amino-acid nucleotides were polymerized to a ribonucleoprotein. This may occur in particular cases, but ribonucleic acid seems also to be concerned in the formation of unconjugated proteins. Amino-acid nucleotides are potential precursors of both proteins and nucleic acids.

The information already available makes it clear that no generalization about the site of protein synthesis within the cell is likely to be true. In some tissues the nucleus synthesizes protein more actively than the cytoplasm; in others synthesis in the microsomal fraction of the cytoplasm predominates. The mitochondria also appear capable of protein synthesis, though some workers have considered that their

part in the process is almost confined to the supply of energy. Ribonuclease has no effect on incorporation of labelled amino acids by mitochondria from liver and muscle (McLean, Cohn, Brandt, & Simpson 1958). This is probably due to the existence (Rendi 1959) within the mitochondria of particles resembling microsomes in size and in their high content of ribonucleic acid. Incorporation of labelled leucine by these particles is sensitive to ribonuclease, in intact mitochondria they are presumably protected from its action. Particles resembling microsomes are also reported in the nucleus (Osawa, Takata, & Hotta, 1957). In green tissues chloroplasts are probably a major seat of protein synthesis. Incorporation of  $C^{14}$  labelled glycine and perhaps more significantly a slight increase (3.2 per cent) in the total protein have been reported for isolated chloroplasts from tobacco leaves. Mitochondria showed a high rate of glycine incorporation, but no increase in total protein (Sisakyan & Filippovich 1957). The authors attributed their findings with mitochondria to simultaneous hydrolysis and synthesis, incorporation of glycine may also reflect some process not implying a net synthesis of protein. Incorporation of  $C^{14}$  labelled leucine and valine into proteins of tobacco leaf disks and isolated chloroplasts was considerably greater in the light than in the dark (Stephenson, Thimann, & Zamcenik, 1956).

Marston (1923-1926) suggested that water poor phases at the surface of lipid elements of the mitochondria provided suitable conditions for protein synthesis. Robertson (1926) stressed the orientating and concentrating effect of the lipid-water interface in syntheses whose substrates have, like amino acids, both hydrophilic and lipophilic groups. Hendler (1958) and Hunter, Brookes, Crathorn, & Butler (1959) also found lipids to be involved in protein synthesis by animal tissues and bacteria. Any intracellular structure is surrounded (Devaux, 1903-1930) by a film formed of oriented heteropolar monomolecular layers, a structure likely to favour differing reactions in proximity to one another. The structural features of such particles as microsomes and mitochondria must be significant in co-ordinating the many contrasting reactions that proceed smoothly in the living cell and are disorganized at its death.

The only conclusion possible at present is that most organs and tissues can synthesize protein some much more actively than others. On a sub-cellular scale a similar position applies, the nucleolus and the microsomes appear to specialize in protein formation, but it occurs also in other intra-cellular structures.

## I. Protein Synthesis in Cell-free Systems

It is clear from the preceding discussion that in general protein synthesis requires the integrity of intracellular structures and possibly of the cell as a whole. Reproducible synthesis in cell-free preparations and still more in homogeneous aqueous solutions would be convenient in studying the process. Such synthesis may be difficult to obtain experimentally, and of dubious relevance to the natural process if it is achieved. Prospects of success are naturally greater with cell-free but still complex preparations containing particles such as mitochondria or microsomes than with clear solutions.

Protein synthesis has been reported in various systems of this type. Khesin (1953) stated that intracellular granules from pigeon pancreas cells retained the ability to synthesize amylase for 20 minutes after disruption of the cells. The observed increases were small but apparently consistent in preparations supplied with adenosine triphosphate,  $\alpha$ -ketoglutarate, and all the amino-acids contained in the invertase molecule. Khesin, Petrashkaite, Tolyushis, & Paulauskaite (1955) obtained from pigeon pancreas and rat liver intracellular granules resembling mitochondria in size but distinguished from them by lower density and a higher content of ribonucleic acid. These granules were found to increase their total protein content (determined by precipitation with trichloroacetic acid) for twenty minutes after isolation; thereafter any continuing synthesis was outstripped by hydrolysis. Synthesis required the provision of all protein amino-acids and also a medium in which mitochondria had been incubated with adenosine triphosphate and a respiratory substrate. The mitochondria when supplied with adenosine triphosphate form some substance required for protein synthesis; its nature is unknown but labile phosphorus compounds seem to be excluded. Webster (1955) reported brief experiments in which a particulate preparation from pea roots incorporated amino-acids into protein. This work was described in greater detail by Webster & Johnston (1955). Particles sedimented at 40,000 incorporated  $C^{14}$ -labelled glutamate, the rate of incorporation being increased by ribonucleic acid and to a greater extent by mixtures of nucleotides, nucleosides, purines, and pyrimidines. Bates, Craddock & Simpson (1958) reported the incorporation of labelled amino-acids into cytochrome *c* in mitochondria from rat liver. Campbell, Greengard & Kernot (1958) stated in a brief report that amino-acids were incorporated into a firmly-bound protein in isolated liver microsomes incubated

in cell sap Lund (1959) reported a synthesis of aldolase in isolated microsomes from the scutellum of *Zea mays*

In these experiments increases in total protein where measured were mostly very small In some cases protein formation was deduced from measurements of enzymatic activity This may be misleading as the final stage in the formation of an enzyme molecule may be a minor change in a protein precursor no net synthesis of protein being involved Reduced activity of an inhibitor could also simulate synthesis of an enzyme Labelling of protein by incorporated amino acids is suspect as a criterion of synthesis unless there is convincing evidence of increased total protein in the experimental system Numerous exchange reactions between proteins and free amino acids are known where incorporation involves no net synthesis of protein Bates & Simpson (1959) provided good evidence for the synthesis in calf liver mitochondria of an individual protein cytochrome c A net synthesis of protein occurred during the experiments Labelled lysine and valine were found after partial hydrolysis of the protein at the expected locations in a known sequence of amino acid residues

## J. Control of Protein Synthesis

Genetic determination of specific and individual features in the development of an organism may plausibly be supposed to involve some form of control over protein synthesis This proposition is more dogmatically expressed by the well known 'one gene one enzyme' hypothesis which in its extreme form may appear a *reductio ad absurdum* but nevertheless probably contains an important element of reality Genetic control seems to operate largely through deoxyribonucleic acid as indicated by its prominence in chromosomes in the transformation of bacteria from one strain to another (Avery McCleod & McCarty 1944, Belozerski Spirin, Kudlai & Skavronskaya 1955) and in the part of the bacteriophage particle that enters the host cell (Hershey & Chase 1952) The experimental evidence at present available suggests however that ribonucleic acids affect protein synthesis more directly Specific deoxyribonucleic acids in the nucleus may control the formation of specific ribonucleic acids which in turn induce specific proteins, but this is still speculative It is not yet known with what degree of precision protein molecules are multiplied within a species or indeed within an individual organism Some proteins of comparatively low molecular weight, e.g. the insulins of several mammals appear to have a definite constitution within a species, and to vary slightly in their component

amino-acids between species. The best methods now available for the separation and analysis of large protein molecules cannot determine whether the properties of a particular protein imply uniformity on the molecular scale or are the statistical resultant of molecules varying in size, composition, or both. It is possible that the activity of enzymes and other biologically active proteins depends upon the structure of comparatively small active centres, together with the configuration of the amino-acid residues in their immediate neighbourhood. The rest of the molecule might then be regarded as an inert carrier whose composition could vary within limits defined by such factors as its size and shape, and the balance of amino, carboxyl, and other reactive groups in the side-chains. It is sometimes assumed, tacitly at least, that proteins can in principle if not yet in practice be defined by unequivocal structures as rigidly determined as those of, say, amino-acids. This assumption should be recognized as such, especially in the absence of experimental methods sensitive enough to confirm or deny it for large protein molecules.

The concept that the bodies of organisms are built of individual substances formed by the precise replication of identical molecules has led to great progress in the last hundred years, culminating in the determination of precise structures for compounds as complex as insulin and vitamin B<sub>12</sub>. A different approach to molecular individuality may be appropriate for proteins, nucleic acids, and highly polymerized substances of simpler composition such as starch, cellulose, chitin, and the polymers of glutamic acid produced by some bacteria. These matters are of little immediate importance in studies of protein composition, where for some time the purification of compounds for analysis is likely to be a limiting factor, except in so far as they raise the question whether the concept of chemical purity is applicable to protein preparations of high molecular weight. Theoretical discussions of specificity in protein synthesis and its relation to the transmission and realization of hereditary characters are, however, affected by considerations of molecular individuality. Mechanisms adequate to determine the formation of specific configurations involving a few amino-acid residues, and to install them on a large protein carrier molecule of structure varying within defined limits, are already difficult to visualize. The difficulty is likely to be much greater if we postulate rigid specification and control of the complete structure in the molecules of numerous large proteins within each organism.

Speculation has been very active concerning possible ways whereby

pre-existing molecules of ribonucleic acid may determine the formation of specific proteins. This theoretical and speculative work, though a valuable stimulus and guide to experimental studies, has not yet clarified the relations between nucleic acids and protein synthesis. Nucleic acids are simpler in structure than proteins as they have fewer components. The main components of ribonucleic acids are the purines adenine and guanine, and the pyrimidines cytosine and uracil, three of these bases occur in deoxyribonucleic acids, but another pyrimidine, thymine, replaces uracil. Many nucleic acids appear to contain only four bases; some also contain 5-methyleytosine, and other substituted purines and pyrimidines have been detected, usually in small amounts. The high molecular weights (of the order of  $10^5$  to  $10^7$ ) now attributed to nucleic acids (Signer, Caspersson, & Haummarston, 1938; Cohen & Stanley, 1942; Katz, 1952) imply the potential existence of very numerous individual compounds, as many perhaps as the actually existing proteins though fewer than the theoretically possible protein molecules.

Much attention has been given to variants of the 'template' hypothesis, which proposes that a pre existing structure serves as a mould, model, or matrix determining the amino acid sequence in a newly synthesized protein. This pre-existing structure was in some early versions of the 'template' hypothesis supposed to be a protein transmitted genetically, but is now usually held to be a ribonucleic acid. Caldwell & Hinshelwood (1950) suggested, for instance, that amino-acids condense on a nucleic acid molecule in a sequence strictly determined by its structure. Ways in which the varying sequences of nucleotides in a nucleic acid could specify an individual amino acid have been considered theoretically (Gamow, Rieh, & Yčas, 1955; Brenner, 1957; Crick, Griffith, & Orgel, 1957). It appears that a 'code' using two nucleotides to specify an amino acid would give far too few choices, while the number of different amino-acid sequences already known is more than could be distinguished by overlapping groups of three nucleotides (Brenner, 1957). Non-overlapping groups of three could, however, constitute a code for twenty, and only twenty, amino acids (Crick *et al.*, 1957). This agreement with the number of amino acids usually found in proteins is interesting; but other amino-acids do occur in some proteins and would have to be provided for in a general 'coding' system. Some, such as hydroxyproline and hydroxylysine, may be formed from 'standard' amino acids after their incorporation, but this can hardly be true for some unusual amino acids Bonner (1959).



pointed out that on current 'coding' theories the ribonucleic acid in a microsome could determine the sequence of only a few hundred amino-acid residues, and suggested that individual microsomes synthesize a single protein. Numerous types of microsome, morphologically similar but functionally specialized to form different proteins, might exist in a single cell.

The chemical and enzymatic basis for such a 'coding' system is largely hypothetical: it is probable that the units condensing on a nucleic acid 'template' would be activated amino-acids, i.e. amino-acid-nucleotide compounds, rather than free amino-acids or peptides. It has been suggested that the nucleotide part of such intermediates in protein synthesis could combine by hydrogen bonds with specific sites on a ribonucleic acid 'template'. It seems possible that a detailed version of these general ideas, which may well represent in outline the means by which specificity is achieved in protein synthesis, will be elaborated and subjected to experimental test in the near future. Protein synthesized in the microsomes appears (Rabinovitz & Olson, 1956, 1959) to be rather firmly bound to ribonucleic acid; this suggests that the newly formed peptide chains are held to nucleic acid by bonds whose rupture involves an energy-requiring reaction. Another observation that hints at further complexities as yet only dimly glimpsed is the apparent association of vitamin B<sub>12</sub> with protein synthesis in isolated preparations (Kolor & Roberts, 1957; Wagle, Mehta, & Johnson, 1957) and intact animals (Gokhale & Punekar, 1959). At present a few stages in protein synthesis seem reasonably well established, notably the preliminary activation of amino-acids and the final stages of synthesis in the microsomes, but much remains to be done before the gaps in the process are understood. The available evidence, moreover, comes largely from animal material and may not reflect the position in plants, especially in the chloroplasts.

Some writers have deduced from the recent emphasis on nucleic acids that these compounds are of primary importance in the growth and development of organisms, with proteins playing a subordinate part. This view is unrealistic; both proteins and nucleic acids appear to be indispensable constituents of organisms, and protein enzymes mediate the synthesis of nucleic acids, as in the bacterial systems studied by Grunberg-Manago, Ortiz, & Ochoa (1955, 1956) and Kornberg, Lehman, Bessman, & Simms (1956). In these systems both ribonucleic acid and deoxyribonucleic acids are synthesized enzymatically from nucleotides, formed in their turn by a long sequence of enzymatic

reactions from simple precursors such as glycine, carbon dioxide, aspartic acid, and the amide group of glutamine. It is at least a gross over-simplification to consider a nucleic acid *per se* as a self-replicating molecule; replication requires a complex synthetic system provided with the necessary precursors and sources of energy. Proteins and nucleic acids are formed by interlocking and interdependent processes; both classes of compound, being essential in all types of metabolism, are of primary importance for the life of all known organisms. It has been suggested that protein synthesis may be controlled by structures in which nucleic acids are prominent and perhaps dominant constituents, it is, nevertheless, clear that synthesis of the specific nucleotide configurations determining protein structure is itself controlled by protein-containing enzymes. Protein and nucleic acid appear metabolically indispensable to each other, their syntheses are perhaps only separate aspects of a complex system, essential to growth and life, which our experimental and conceptual approach separates into arbitrary divisions.

#### K. Regulation of Protein Synthesis and breakdown

Early work with isotopic tracers (Hevesy, Linderström-Lang, Keston, & Olsen, 1940) indicated a continuous exchange of nitrogen atoms between tissue constituents and nitrogenous substances entering the plant from outside. Similar conclusions were reached for animals (Foster, Schoenheimer, & Rittenberg, 1939; Shemin & Rittenberg, 1944). The comparatively steady protein content of mature leaves is therefore attributed to a dynamic equilibrium between synthesis and breakdown, as suggested by Borodin (1876). In *Escherichia coli* synthesis of the adaptive enzyme  $\beta$  galactosidase and other proteins is stated (Manson, 1953; Monod & Cohn, 1953; Hogness, Cohn, & Monod, 1955) to be essentially irreversible. Nitrogen in protein and ribonucleic acid in the yeast *Torulopsis utilis* appears to be permanently removed from general metabolism (Chayen, Chayen, & Roberts, 1959). Such results suggest that protein turnover may be very slow, at least in micro-organisms. Data against this view have, however, been reported (Steinberg, Vaughan, & Anfinsen, 1956; Borek, Ponticorvo, & Rittenberg, 1958). Protein turnover seems well established in non-growing micro-organisms. Intense protein synthesis may mask breakdown in growing cultures, making turnover hard to detect. The position in higher plants is obscure and needs more study.

Gardner (1844) studied the effects of light of different colours on the

chlorophyll content of leaves; his results led Berzelius (1845) to conclude that in normal conditions chlorophyll is destroyed and replaced continuously in the leaf. This view is supported by more recent workers, e.g. Turchin, Guminskaya, & Plyshevskaya (1953).

Work on rooted leaves suggests that nitrogen metabolism in attached leaves may be profoundly affected by raw materials or hormones translocated from other parts of the plant. The effects of age and stage of development on protein synthesis in plants (Kursanov & Bryushkova, 1940; Walkley, 1940; Ali-Zade, 1941; Walkley & Petrie, 1941) are consistent with such effects, but no precise mechanisms can be proposed. Kinetin (6-furfurylaminopurine) may be one essential material imported by leaves (Richmond & Lang, 1957). Applied to small areas of detached leaves (*Nicotiana rustica*), it causes a local accumulation of soluble nitrogenous compounds, often accompanied by synthesis of chlorophyll, nucleic acids, and protein (Mothes, Engelbrecht, & Kulayeva, 1959).

Detached fruits of apple (Hulme, 1936, 1948; Turner, 1949) and pear (Kidd, West, Griffiths, & Potter, 1940; Ulrich, 1951) differ markedly from leaves in showing a net protein synthesis, even at the low temperatures used in cool storage. These fruits have, on a fresh-weight basis, a much lower nitrogen content than leaves; a large part (often more than half) of their nitrogen is in soluble compounds. The respiration rate of detached apples shows a characteristic rise at a stage, long after cessation of active growth, known as the 'climacteric' (Kidd & West, 1925). This rise of respiration is associated with synthesis of protein from soluble precursors (Hulme, 1948; 1954a, b; Turner, 1949; Pearson & Robertson, 1953). A metabolic connexion between the increased respiration and the increased protein content seems clear. Robertson & Turner (1951) suggested that increased protein synthesis might increase the content of phosphate acceptors, thus removing phosphate groups more rapidly from respiratory intermediates and increasing the respiration rate. This view was supported (Pearson & Robertson, 1952) by the effect of 2,4-dinitrophenol (DNP) on cut tissue taken from apple fruits before and after the climacteric stage. DNP, which uncouples oxidation and phosphorylation, markedly stimulated the respiration (measured by oxygen uptake) of pre-climacteric fruits. As the fruit passed through the climacteric phase the effect of DNP became steadily less, and was almost completely absent in post-climacteric fruit.

The physiology of fleshy fruits has been studied mainly because of the economic importance of their storage behaviour. Their low content

of nitrogenous substances tends to make them inconvenient material for the study of nitrogen metabolism. They do however raise interesting problems regarding the processes controlling protein synthesis and breakdown and in some respects their slow metabolic tempo may be an advantage in analysing the sequence of events.

## CHAPTER 12

# ALKALOIDS

### A. Definition

Alkaloids are bases containing one or more nitrogen atoms, usually in a heterocyclic ring. Many have profound physiological effects on animals. The great majority occur in flowering plants; a few are known in other groups and in animals. Antibiotics from fungi and bacteria include alkaloids, some chemically very distinct from those of higher plants. There is no clear boundary between alkaloids and other plant bases, particularly the more complex amines. The amines are simpler in structure and somewhat more directly related to amino-acids than are the alkaloids. Some alkaloids are chemically, and probably also metabolically, closer to sterols or terpenes than to amino-acids. The alkaloids are metabolically and structurally heterogeneous; the name, however, is long established, being used in the variant 'alcalinoide' by Bonastre (1824), and is still useful as there is rarely any doubt whether it applies to a particular compound.

### B. General

An enormous literature exists on the chemistry of alkaloids, and on their physiological effects in the animal body. The plants in which they occur attracted early attention, and even among primitive peoples their powerful physiological effects were used to prepare both poisons and remedies for disease. Until recent years the plants examined for alkaloids were traditional sources of drugs or poisons. The alkaloid resources of various floras are now receiving more systematic study; interest is still largely concentrated on families and genera long recognized as alkaloidal. Traditional alkaloids important in modern medicine include atropine, caffeine, cocaine, codeine, emetine, ephedrine, ergometrine, morphine, and quinine. Some new alkaloids have attained medical prominence; those of curare, an arrow poison produced by primitive tribes in South America, form a good example. Great interest was aroused by the discovery (Müller, Schlittler, & Bein, 1952) of strong hypotensive and sedative properties in reserpine, found in several species of *Rauwolfia* and also (Crow & Greet, 1955) in another

member of the Apocynaceae, *Alstonia constricta*. The root of *Rauwolfia serpentina* a traditional drug in Indian and Burmese medicine was shown to contain alkaloids by Eijkman (1887) but had no application in Western medicine until 1952. Since that date a flood of publications indicates the intense interest now taken in alkaloids of *Rauwolfia* and related genera. The results of this work are complex, many species are involved some containing numerous alkaloids. Muller (1957) identified 21 alkaloids in *R. ligustrina* and detected several more. Alkaloid studies in *Rauwolfia* (and in the Apocynaceae generally) are well summarized by Bisset (1958). Several plants in this family have been shown to possess valuable pharmacological properties previously unrecognized. The seeds of *Picralima nitida* contain numerous alkaloids, two components akuammine and akuammidine are very effective local anaesthetics (Raymond Hamet, 1951). The alkaloids in the bark of *Hunteria eburnea* have a powerful and prolonged hypotensive action (Raymond Hamet, 1954). Both these species are native to West and Central Africa.

The investigation of alkaloids still very active in spite of intensive work over the last 150 years, is likely to remain an important branch of chemistry. Even in known alkaloidal families, many species are still untouched chemically. Other families also have scattered alkaloidal members which are more likely to be overlooked. Systematic studies of complete floras to identify their resources in alkaloids and other chemical groups have begun in some countries, e.g. Australia (Webb, 1952) and U.S.S.R. (Sokolov, 1957). These surveys have already brought to light many new alkaloids, some differing considerably in structure from any previously known. Improved methods of separating alkaloids particularly by chromatography, have also revealed the presence in plants that have long been studied of numerous unsuspected minor alkaloids often but not always structurally related to the main alkaloids.

Known alkaloidal plants belong, in round numbers to 100 families, 500 genera and 1,200 species. About 1,000 alkaloids are known, 400 being fully described chemically (Williamson & Schubert, 1955). Partly described alkaloids are much more numerous. Many names of alkaloids now existing in the chemical literature will certainly be reduced to synonymy when the compounds involved are more thoroughly investigated. So many new alkaloids have been described in recent years that any apparent reduction in the number of named alkaloids seems certain to be more than compensated by new discoveries. Unknown

alkaloidal plants and alkaloids must be numerous, but no estimate of their possible numbers can now be made.

### C. Historical

Sertuerner (1806), an apothecary in the small town of Einbeck in Hanover, isolated morphine, the first alkaloid to be isolated and characterized, from opium (the dried latex from unripe fruits of *Papaver somniferum*). Sertuerner (1806, 1817) described the alkaloid, which he named 'morphium', as capable of forming salts, and compared its chemical nature to that of ammonia. Earlier workers on the chemistry of opium probably obtained morphine more or less mixed with other substances, but described it less clearly. Following this discovery, a series of alkaloids was isolated in the next decade, largely by French chemists. Robiquet (1817) showed that opium contained a second distinct alkaloid, narcotine. Pelletier & Magendie (1817) isolated a base which they named emetine from the rhizome of *Uragoga ipecacuanha*, a South American drug investigated earlier by Henry (1806). Pelletier & Caventou (1819) isolated strychnine from several species of *Strychnos*; soon afterwards (Pelletier & Caventou, 1820b) they isolated quinine and cinchonine from cinchona bark; they considered the alkaloids to occur as salts of quinic acid, isolated earlier as its calcium salt from the bark of several species of *Cinchona* (Vauquelin, 1806). Quinine, cinchonine, and quinic acid were further studied by Henry & Plisson (1827). Cytisine was found in *Laburnum vulgare* by Chevalier & Lassaigue (1818).

Meissner (1819) and Pelletier & Caventou (1820a) isolated veratrine from the seeds of species of *Veratrum*. Desfosses (1820, 1821) found the first sterol alkaloid, which he named solanine, in berries of *Solanum nigrum*. He looked for it also in fruits of potato (*S. tuberosum*) but without success. Desfosses remarked that his base resembled cholesterol very closely. This surprisingly accurate statement was probably a lucky guess, for at that time the structures of solanine and of cholesterol were equally unknown.

Nicotine also was recognized early. Vauquelin (1809a) obtained from tobacco leaves (*Nicotiana tabacum*) an acrid, volatile, colourless, highly toxic liquid soluble in water and in alcohol, which he did not name though he rightly considered it to differ from all others then known in the plant kingdom. This preparation clearly consisted largely of nicotine; the base was isolated, named, and described by Posselt & Reimann (1828). Nicotine was further studied by Henry & Boutron-

Charlard (1836); Melsens (1843) detected it in tobacco smoke; Barral (1847) gave the correct empirical formula.

The atropino group of alkaloids, from *Atropa*, *Datura*, *Hyoscyamus*, and other genera of the Solanaceae, was also studied about this time. Vauquelin (1809b) obtained from *Atropa belladonna* a substance precipitated by tannin, soluble in ethyl alcohol and yielding ammonia on pyrolysis. This was presumably a crude preparation of atropine. Runge (1824) named the base, which was further studied by Brandes (1832). The first reasonably pure preparations were probably obtained by Geiger & Hesse (1833a, b) and by Mein (1833). The correct empirical formula was given by Liebig (1833). Geiger (1833) described hyoscyamine, another alkaloid of this group; in the same paper he described colchicine from *Colchicum* and aconitine from *Aconitum*. Pelletier & Caventou (1820a) had isolated colchicine earlier but supposed it to be identical with veratrine. Geiger (1831) isolated coniine, the very poisonous volatile alkaloid of hemlock (*Conium maculatum*).

Isolation of the active materials of drug and poison plants thus provided a long series of new and well-defined substances for chemical study. Analysis and structural investigations began at once, though the latter developed slowly owing to the complex problems involved and the primitive state of organic chemistry. Dumas & Pelletier (1823), in a paper forming an important landmark in alkaloid chemistry, gave analyses of nine well-characterized bases (brucine, caffeine, cinchonine, emetine, morphine, narcotine, quinine, strychnine, and veratrine). These alkaloids were comparatively easy to isolate; determination of their structures has occupied some of the greatest organic chemists for over a hundred years, and some points are still not settled. Elucidation of alkaloid structures has provided some of the greatest difficulties and triumphs of organic chemistry; the molecules are not particularly large, but some alkaloids with twenty or thirty carbon atoms are structurally very complicated.

Liebig (1831) and Regnault (1838) applied new and more accurate analytical methods to determine the composition of alkaloids and of numerous salts prepared from them. These chemists, and also Matteucci (1833), put forward the idea that alkaloid structures were based on substituted ammonia molecules. This concept was furthered by the brilliant work of Wurtz (1850) and Hofmann (1850) on the constitution of organic primary, secondary, and tertiary amines. The recognition (Gerhardt, 1842) of the comparatively simple base quinoline ( $C_8H_7N$ ) as a breakdown product, and therefore a putative structural component,



of quinine was an important development. The discovery (Anderson, 1851) of pyridine among the products of destructive distillation of bones also influenced alkaloid chemistry, as pyridine may be considered the mother substance of a whole group of alkaloids. In spite of all this painstaking, competent, and sometimes brilliant work it was long before the structure of an alkaloid was established and confirmed by synthesis; the feat was first accomplished for coniine (Schiff, 1870; Ladenburg, 1889).

#### D. Distribution of alkaloidal species in the plant kingdom

Alkaloidal plants are scattered erratically through the plant kingdom. They appear to be rare or absent among algae, whose chemistry is, however, still very incompletely known. Fungi, lichens, and bacteria (particularly actinomyces) include alkaloidal species;

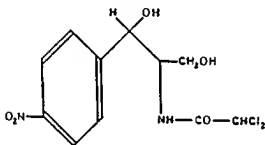
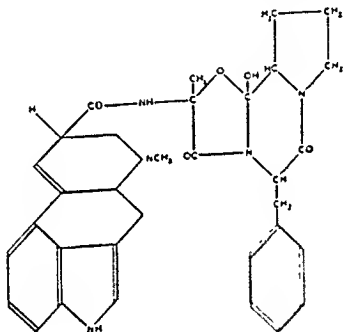


FIG. 61.

some of the antibiotics brought into medical use in recent years are true alkaloids, e.g. chloromycetin (chloramphenicol) from *Streptomyces venezuelae* (Fig. 61). Jaconine, an alkaloid from *Senecio jacobaea* (Compositae), also contains chlorine (Bradbury & Culvenor, 1954). The alkaloids of ergot (*Claviceps purpurea*, a fungal parasite of grasses) have long been noted both for their clinical value and for the complexity of their structure. As an example, we may mention ergotamine (Fig. 62), in which a nucleus formed by the fusion of indole and isoquinoline rings is joined to a cyclic polypeptide (Stoll, Hofmann, & Becker, 1944; Stoll & Hofmann, 1950) of the type suggested by Wrinch (1937a, b) as a model for proteins, in which, however, it has not yet been found. The ferns, a numerous group of plants spread all over the world, appear to lack alkaloids. Two smaller groups of vascular cryptogams, the lycopods (*Lycopodium* spp.) and horsetails (*Equisetum* spp.), contain complex alkaloids whose structures are incompletely known (Manske & Marion, 1942; Eugster, Griot, & Karrer, 1953). Few alkaloids are

known from the gymnosperms. Ephedrine ( $\beta$ -phenyl-hydroxyisopropyl-N-methylamine) was first isolated (Miura, 1887) from the traditional Chinese drug ma huang, a product of several species of *Ephedra* (Gnetaceae). The yew (*Taxus baccata*) contains ephedrine (Gulland & Virden, 1931), known also from several flowering plants, including *Roemeria refracta* (Papaveraceae) (Kononova, Yunusov, & Orekhov, 1939). The yew has a further alkaloid, taxine, of more complex structure than ephedrine (Marmé, 1876; Amato & Capparelli, 1880; Callow,



Ergotamine

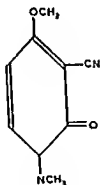
FIG. 62.

Gulland, & Virden, 1931). Pindine, a piperidine alkaloid of comparatively simple structure, occurs in *Pinus jeffreyi*, *P. attenuata*, and *P. torreyana* (Tallent & Horning, 1936).

Among the angiosperms there are a few families, e.g. Papaveraceae, whose tested species are all alkaloidal. Other typically alkaloidal families include Amaryllidaceae, Apocynaceae, Labiaceae, Menispermaceae, Rutaceae, and Solanaceae. Many other families have a few such product genera and a much larger number of genera and species producing alkaloids. Examples of this type are the grasses (*Citrus* and *Pinus* (Palmar), Convolvulaceae, Ranunculaceae, Compositae, and Ericaceae). The last family has in the past been considered much more

but lately Russian workers have isolated and studied a large number of alkaloids from the genera *Anabasis*, *Arthrophytum*, *Girgensohnia*, *Halostachys*, *Petrosimonia*, and *Salsola*.

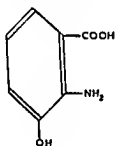
Some alkaloids occur in several species widely separated systematically, others are known only from a single species or genus. Some



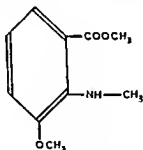
Ricine

FIG. 63.

alkaloids of simple structure have a very restricted known distribution, e.g. ricinine (Fig. 63) from *Ricinus communis*, damascenine (Fig. 64) from *Nigella damascena*, and salsoline (Fig. 65) from the genus *Salsola*. The apparently restricted occurrence of these simple compounds is surprising, especially as they are closely related to common metabolites,



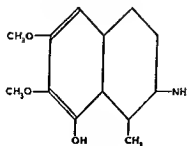
3-Hydroxyanthranilic acid



Damascenine

FIG. 64.

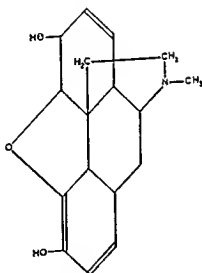
e.g. damascenine to 3-hydroxyanthranilic acid, a breakdown product of tryptophan. Morphine (Fig. 66), long known only from *Papaver somniferum*, has been detected in *P. setigerum* (Kleinschmidt, 1958). Other alkaloids of the morphine type occur in *Papaver*, and also in *Sinomenium acutum* and *S. diversifolium* (Menispermaceae) (Holmes, 1952), *Rauwolfia serpentina* (Apocynaceae) (Hofmann, 1954) and



Salsoline

FIG. 65.

*Strychnos melinoniana* (Loganiaceae) (Bachli, Vamvacas, Schmid, & Karrer, 1957). Alkaloids of the cularine type, distinguished by a 7-membered ring containing oxygen in a diphenyl ether linkage are known only from two genera of Fumariaceae, *Corydalis* and *Dicentra* (Manske, 1940, 1950).



Morphine

FIG. 66.

Nicotine occurs throughout the genus *Nicotiana*; most of its 50 species have been examined chemically without the discovery of any lacking the alkaloid. *Nicotiana* was long supposed to be the only genus to produce nicotine, early reports of its presence in *Cannabis sativa* (Procobrazhenski, 1876) and in *Duboisia hopwoodii* (Petit, 1879) being generally disregarded. The data cited by these workers as identification

of nicotine were not completely convincing by modern standards, moreover, the hashish analysed by Preobrazhenski (1876), though no doubt prepared mainly from *Cannabis*, may have contained tobacco, which is sometimes added to the drug. Nicotine seems not to have been reported again in *Cannabis*, its presence in *Duboisia hopwoodii* is amply confirmed (Rothera, 1910, Bottomley, Nottle, & White, 1945, Trautner & Neufeld, 1946), though it is replaced by normicotine in some samples of this species (Hicks & Le Messurier, 1935, Späth, Hicks, & Zajic, 1935, Hicks & Sinclair, 1947).

More recent work has clearly shown that nicotine is not restricted to any narrow systematic group. In the family Solanaceae, to which

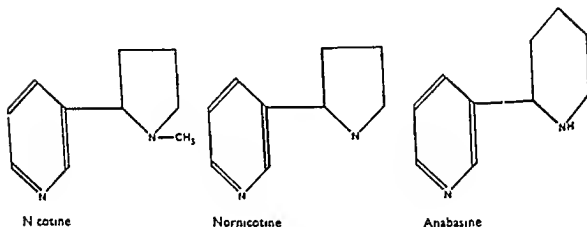
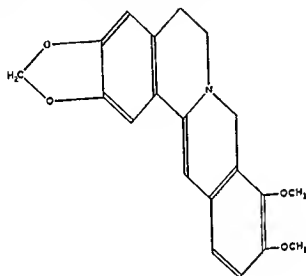


FIG 67

*Nicotiana* and *Duboisia* belong traces of nicotine occur (Mothes, 1953, Wahl, 1952) in several species of *Atropa*, *Datura*, and *Solanum*, it also occurs in some samples of *Duboisia myoporoides* (Hills, Bottomley, & Mortimer, 1953, Mortimer & Wilkinson, 1957) and in *Withania somnifera* (Majumdar, 1955). Nicotine, now known from two genera of vascular cryptogams (*Equisetum* Manske & Marion 1942, Karrer, Eugster, & Patel, 1949, Eugster, Griot & Karrer 1953 and *Lycopodium* Manske & Marion, 1942), is not reported in ferns, gymnosperms, or monocotyledons, but occurs in several unrelated families scattered through the dicotyledons. Nicotine containing species include Asclepiadaceae *Aclepias syriaca* (Marion 1939), Compositae *Eclipta alba* (Pal & Narasimham, 1943), *Zinnia elegans* (Schröter, 1955), Crassulaceae *Sedum acre* (Marion 1945), *Sempervivum arachnoideum* (Paris & Friot, 1959), Leguminosae *Mucuna pruriens* (Majumdar & Paul, 1954). In most of these species nicotine is a minor component, apart

from some species of *Nicotiana*, it is a major alkaloid only in *Zinnia elegans* and in some samples of *Duboisia hopwoodii* and *D. myoporoides*; in the last two species the closely related bases nor nicotine and anabasine may replace it. Anabasine occurs in species of *Nicotiana*, usually as a minor alkaloid. It is the major alkaloid of the quite unrelated genus *Anabasis* (Chenopodiaceae) (Orekhov & Menshikov, 1931) (Fig. 67). Nicotine, once supposed to be restricted to a single genus, is now known from many unrelated plants. This change suggests that any conclusions based on the known distribution of alkaloids among plant



Berberine

FIG. 68.

species must be regarded as highly tentative, particularly for minor alkaloids. Some alkaloids occurring in several different families are, like nicotine, of comparatively simple structure. Other more complex alkaloids are also widely distributed. Berberine (Fig. 68) is recorded from several families. Bebeerine (Fig. 69) (not to be confused with berberine), another complex base, is known from *Nectandra* (Lauraceae), *Buxus* (Buxaceae), and several genera (*Cissampelos*, *Chondodendron*, *Pleogyne*) of Menispermaceae (MacLagan, 1843; Scholtz, 1896; King, 1939, 1940; Anet, F. A. L., Hughes, & Ritchie, 1950). Quinidine, long known only from South American species of *Cinchona* (Rubiaceae), is recorded (Buzas, Osowiecki, & Régnier, 1959) from the bark of *Enantia polycarpa*, an African species belonging to Annonaceae, a family quite unrelated to Rubiaceae.

The three species of *Duboisia* (Solanaceae), provide an interesting example of variability of alkaloids within a genus, and also within individual species. *D. hopwoodii* grows in arid areas of central Australia. Its alkaloids resemble those of the genus *Nicotiana*, nicotine being the main alkaloid in some samples and nornicotine in others (Bottomley, Nettle, & White, 1945; Hicks & Le Messurier, 1935). The other two species, *D. myoporoides* and *D. leichhardtii*, have alkaloids mainly of the mydriatic (tropane) type. These two species are now major commercial sources of mydriatic alkaloids, and have received some biochemical and physiological study. *D. myoporoides* occurs in a long narrow area along most of the east coast of Australia, and also in New Caledonia. Even within a single tree the alkaloids may vary considerably at different times, but in general scopolamine predominates in *D. myoporoides* in the northern part of its range and hyoscyamine in the southern part. The boundary between these two types is marked approximately by the town of Gosford, New South Wales. Hyoscyamine is the main alkaloid of *D. leichhardtii*, which has a restricted area in south-east Queensland. Some trees show a fairly constant alkaloidal composition over the year; in others it fluctuates violently. Some trees of both species contain appreciable amounts of atropine and norhyoscyamine; tigloidine and valericidine also occur in *D. myoporoides*. These data are due mainly to Hills and his co-workers (Hills, Trautner, & Rodwell, 1945a, b; Hills & Rodwell, 1946; Trautner, 1947; Hills, Bottomley, & Mortimer, 1954). Earlier workers also noted variability in the alkaloids of *Duboisia*. Schmidt (1890), unlike Ladenburg (1880) who found only hyoscyamine, recorded both hyoscyamine and hyoscyne in *Duboisia* leaves. Petrie (1917a, b) noted the variability of the alkaloids in *D. myoporoides*, and recorded norhyoscyamine in *D. leichhardtii*.

Von Mueller & Rummel (1879) isolated from leaves and twigs of *Duboisia myoporoides* of unstated but presumably Australian origin a volatile alkaloid resembling nicotine but considered to be distinct from it. Their material may have been nicotine mixed with other volatile alkaloids. This early observation of tobacco-type alkaloids in *D. myoporoides* seems to have attracted little attention, but has been extended by more recent work. A New Caledonian form of the species contains scopolamine, nicotine, and nornicotine (Hills, Bottomley, & Mortimer, 1953); an Australian form produces (Mortimer, 1957; Mortimer & Wilkinson, 1957) scopolamine, nicotine, anabasine, and isopelletierine, otherwise recorded only in the pomegranate (*Punica granatum*) (Tanret, 1878) and in *Sedum acre* (Crassulaceae) (Frank,

1958). The varied physiological forms of this species thus form a wide range of alkaloids in the tropane and pyridyl series.

### E. Alkaloids in the animal kingdom

The few compounds of animal origin which can be classed as alkaloids stand in contrast to the vast number known from plants. Bufotenine (5-hydroxyindolyl-ethyl-dimethylamine) was first characterized by Wieland, Konz, & Mittasch (1934), who isolated it from the poisonous skin secretions of *Bufo communis* and other toads. Later it was found in *Amanita mappa* and other higher fungi (Wieland & Motzel, 1953), and in the seeds of *Piptadenia peregrina* (Leguminosae), where it forms almost 1 per cent of the dry weight (Stromberg, 1954). This plant was used as a ceremonial narcotic snuff in Haiti when Europeans first arrived there late in the fifteenth century. The poisonous secretion of the European salamanders *Salamandra maculosa* and *S. atra* contains an alkaloid samandarine (Zaleski, 1866; Schöpf & Braun, 1934; Schöpf & Koch, 1942; Schöpf, Blödorn, Klein, & Seitz, 1950). Its structure, not fully determined, is more complicated than that of bufotenine and contains the oxazolidine ring, not known from any other natural product.

### F. Localization of alkaloids in the plant

Most of the information available on this subject comes from microchemical studies using a wide range of reagents to detect alkaloids in plant tissues. Schaarschmidt (1884), an early worker in this field, studied the distribution of solanine in species of *Solanum*. Votchal (1887, 1888, 1889) worked on the same alkaloid in *Solanum tuberosum* and *S. dulcamara*. It may be mentioned that the name of this author is spelt as given above when transliterated from the Cyrillic script by the method now current. Several variants (Woczal, Wotschal, Wotschall, Wothtschall) appear on his papers and in references to them. His studies were very thorough and he gave, especially in his Russian papers, much information on earlier work with solanine. About the same time a Belgian group began a long study (Errera, Maistriau, & Clautriau, 1887; Clautriau, 1889, 1894; Molle, 1895; and many other publications) on the distribution of alkaloids within the plant. Many species were studied, Molle (1895) including in his work on the Solanaceae *Atropa belladonna*, *Brufelsia americana*, *Datura stramonium*, *Hyoscyamus niger*, *Nicandra physaloides*, *Nicotiana tabacum*, *Petunia violacea*, *Physalis alkekengi*, *Salpiglossis sinuata*, *Scopolia japonica*, *Solanum*



*dulcamara*, and *S. tuberosum*. The results of these investigations, extended by later workers (e.g. Klein & Sonnleitner, 1929; Chaze, 1927, 1929; James, 1946a), showed considerable variation in behaviour between the alkaloids of different species. The histochemical methods used rarely if ever identified individual alkaloids, giving only the distribution of total alkaloid within a tissue.

In most species alkaloids are particularly prominent in actively growing tissues. In *Ricinus communis*, for instance, ricinine is formed actively in young plants and in developing organs of older plants; its synthesis seems to be confined to growing tissues (Bogdashevskaya, 1952). In barley seedlings hordenine occurs mainly in the meristematic cells of the root tip (Reilhes, 1936). In meristems of solanaceous plants alkaloids form inside incipient vacuoles and are held later in the vacuoles of storage tissue (James, 1946a). The embryo and endosperm in these species are free of alkaloid (though it accumulates in dead tissues of the seed-coat); alkaloids appear very early in germination (Molle, 1895; James, 1946b). Similar results were noted for several other species of Solanaceae by the Brussels group, who also recorded a complete absence of alkaloid from the seeds (including the seed-coat) in *Nicotiana tabacum*, *Papaver somniferum*, and *Physalis alkekengi*. Seeds of *Solanum dulcamara*, *S. nigrum*, and *S. tuberosum* contain very little solanine (Votchal, 1889), though it is abundant in the unripe fruits. In some species, e.g. *Lupinus luteus*, *Veratrum sabadilla* (*Schoenocaulon officinale*), *Physostigma venenosum* (Jobst & Hesse, 1864), alkaloids accumulate more in the seeds than in other parts of the plant. Unripe seeds of *Nicotiana tabacum* contain nicotine, which disappears as they ripen (Ilyin, 1934). In *Nicotiana rustica*, however, the nicotine content increases as the seeds ripen (Mothes & Romeike, 1951). *Papaver somniferum* has morphine in the leaves and roots in the earlier stages of development, but the capsule, with the upper part of the stem, contains all the alkaloid of the mature plant (Hills, 1945); the seeds are free of alkaloids (Annett, 1920).

Alkaloids are usually present throughout young, actively growing tissues. Later they tend to be localized in particular tissues, and to disappear elsewhere. Tissues retaining alkaloids at this stage include epidermis, phloem parenchyma, and xylem parenchyma. In roots the alkaloids are often deposited in the outer layers of cells, which become the root bark. Root bark is, therefore, a rich source of alkaloids in some species. In some species of *Berberis* (Chatterjee, 1943) alkaloids are deposited mainly in dead cells of the stem bark. Lotsy (1897) found that

in *Cinchona calisaya*, *C. ledgeriana*, and *C. succirubra* alkaloids were absent from young meristematic parts but accumulated in the bark.

Some changes in alkaloid type between different parts of the plant have been recorded. Cromwell (1956) found that in young leaves and other vegetative tissues of *Conium maculatum* the main alkaloid was  $\gamma$ -coniceine; in flowers and young fruits this base was replaced by coniine and *N*-methyleconiine, the latter predominating in mature fruits. *Colchicum speciosum* contains, besides colchicine, another alkaloid colchicerine (Beer, 1949). Old bulbs in autumn contain only colchicerine, and young bulbs during the period of active growth in spring contain only colchicine. The change-over from colchicine to colchicerine begins at the start of seed-ripening and is complete when the bulbs enter the annual dormant period in late summer (Karapetyan, 1950).

### G. The site of alkaloid formation in the plant

In early physiological studies of the formation of alkaloids it was often assumed that they were produced in the leaves, which seemed fitted for this rôle, being metabolically very active organs and in many species having a high alkaloid content. It has since been realized that the roots are also active metabolically, and that alkaloids are not necessarily formed at the sites where they accumulate. These general ideas are consistent with alkaloid formation in roots, for which there is also more specific and in some cases conclusive evidence.

Much of this evidence is derived from grafting experiments. The use of this technique in alkaloid investigations goes back to Strashurger (1885); other pioneers in the field were Grafe & Linsbauer (1906), Meyer & Schmidt (1907), and Javillier (1910). The species used were generally members of the Solanaceae, and many intergeneric grafts were tried with varying degrees of success. The general belief that alkaloids were formed in the shoot led to the use of scions from alkaloidal species on alkaloid-free stocks. The expected transfer of alkaloids from scion to stock was rarely observed. Both stock and scion often had little alkaloid. These inconclusive experiments were also affected by metabolic interactions between stock and scion. Such interactions are not clearly understood, but their existence is shown by much empirical observation with fruit trees, and turned to advantage in selecting stocks for specific purposes, e.g. to dwarf the scion.

More recent studies have shown that in many Solanaceae the root is the main seat of alkaloid formation. Many workers made grafts in

which alkaloids characteristic of the stock appeared in the scion. In *Atropa* grafted on a tomato stock, for instance, the scion is free of mydriatic alkaloids, but in the reciprocal graft they appear in tomato grafted on an *Atropa* stock. Similar results with many combinations of stock and scion established that in *Nicotiana* species and the mydriatic Solanaceae the stock determines the alkaloid content of the whole grafted plant. Workers contributing to this advance included Daniel & Potel (1925), Hasegawa (1937), Shmuk, Kostov, & Borozdina (1939), Kerkis & Pigulevskaya (1941), Dawson (1941), Hieke (1942), Mothes & Hieke (1943), Cromwell (1943a), Hills, Trautner, & Rodwell (1945b), Vincent & Dulucq-Mathou (1946), Wilson (1952a, b), and Ilyin (1955). Schröter (1955) grafted *Zinnia elegans* (Compositae) on a tobacco stock. This species is the only composite known to contain appreciable amounts of nicotine, and this chemical similarity may explain the unexpected success of the graft. *Nicotiana affinis* also flowers (Parcot, 1922) as a scion on the very different species *Amarantus caudatus*. The work on grafted plants and its implications for the physiology of alkaloids in the plant have been summarized by Dawson (1948), Ilyin (1949), and Mothes (1955).

Leaves free from the alkaloids normally contained in their species can be obtained from scions grown on stocks of other species. Alkaloids appear in these leaves if they are caused to form roots. This was shown by Ilyin (1948) with leaves from scions of *Nicotiana tabacum* grafted to tomato stocks, and by Lashuk (1948) with leaves of *Nicotiana sylvestris* grown in the same way. Lashuk (1948) sampled leaves at several different stages after rooting was established, and at each time of sampling analysed separately four parts of the leaves at increasing distances from the petiolar (rooted) end. Nicotine accumulated steadily at the petiolar end of the leaf until the observations were terminated seventy days after the leaves had rooted. In the middle of the leaf nicotine accumulated to a rather smaller extent, but in the part of the leaf farthest from the petiole comparatively little was found, and the amounts present decreased towards the end of the experiment. Very little normicotine was found in the roots or at the petiolar end of the leaf, but it accumulated in large amounts towards the tip of the leaf. These results suggest that in *N. sylvestris* nicotine formed in the roots is translocated to the leaves and there demethylated to normicotine. Demethylation of nicotine in this species is stated to predominate in aging tissues (Mothes, Engelbrecht, Tschöpe, & Hutschenreuter-Treffitz, 1957).

Nornicotine occurs together with nicotine in the roots of several species of *Nicotiana*. Its presence in the root does not exclude the possibility that it is formed only in the shoot, and transported downwards to the root. Excised roots cultivated in sterile conditions (Schröter & Engelbrecht, 1957), can, however, produce nornicotine, together with nicotine and anabasine, in *Nicotiana glauca*, *N. paniculata*, *N. rustica*, and *N. sylvestris*. Schröter (1957) infiltrated nicotine labelled with  $C^{14}$  into detached leaves and shoots of *N. glauca*, some nicotine was converted to nornicotine but more to anabasine. In grafting experiments Pyriki & Muller (1957) also found evidence for the production of nornicotine in the roots of several *Nicotiana* species. Kuzin & Merenova (1952) showed that in leaves of tobacco supplied in the dark with  $C^{14}$  labelled carbon dioxide, the pyridino methyl group of nicotine contained  $C^{14}$ , transmethylation must therefore occur in the leaves. Some workers (Dawson, 1942a, Ilyin, 1948, Mashkovtsev & Sirotenko, 1951) found small amounts of nicotine in leaves of *Nicotiana* scions on tomato stocks. This may be due to traces of nicotine formed normally in tomato (Wahl, 1932). Solt (1957), however, demonstrated a limited synthesis of nicotine from tritium labelled nicotine acid in the shoot of *Nicotiana tabacum*.

Isolated roots in sterile media can also form alkaloids. Since roots cultured in this way have no connexion with a shoot system, they require an energy source, and usually also essential growth factors, which the shoot supplies to the root in the intact plant. Alkaloid synthesis by isolated roots occurs in *Nicotiana* (Dawson, 1942b), *Datura* (Peacock, Leyerle & Dawson, 1944, Stenstra, 1954), *Hyoscyamus* (Telle & Gautheret, 1947), and *Atropa* (Reinouts van Haga, 1957). The presence of substantial amounts of alkaloids in the bleeding sap of decapitated plants provides further evidence for their synthesis in the root (Dawson, 1942a, Hiehe, 1942, Reuter, 1956). Direct histochemical observations show that alkaloids appear in young roots formed by alkaloid free embryos (Chaze, 1932, James, 1946b, Schmid & Serrano, 1948, Fardy, Cuzin, & Schwartz, 1953). Nicotine synthesis appears to be confined to actively growing roots. Rooted leaves of *Nicotiana rustica* greatly increase their nicotine content if the roots are repeatedly cut back to stimulate meristematic activity (Mothes, Engelbrecht, Tschöpe, & Hutschenreuter-Truffitz, 1957). Boron deficiency causes excessive branching of roots in *N. tabacum* producing a much larger proportion of young root tissue than in normal plants. The nicotine content of boron deficient plants is very high up to four times that of control

plants on a dry-weight basis. This increased production of nicotine may reasonably be attributed to the greater amount of meristematic root tissue (Steinberg, 1955).

The root is often, but not always, the main site of alkaloid synthesis. Reciprocal grafting experiments indicate that in tomato, potato, and *Solanum demissum* the scion rather than the stock controls the sterol alkaloids and their glycosides (Prokoshov, Petrochenko, Ilyin, Baranova, & Lehedeva, 1952; Guseva & Paseshnichenko, 1958). In *Nicotiana glauca* both root and shoot seem to form anabasine independently (Shmuk, Kostov, & Borozdina, 1939; Dawson, 1944; Lashuk, 1948; Leete, 1958a). There is also evidence for the formation of alkaloids in the shoots of *Berberis darwinii* (Cromwell, 1933) and of *Conium maculatum* (Cromwell, 1956). Norpseudo-ephedrine appears to be formed in the shoot of *Catha edulis* (Leete, 1958b), and ephedrine in that of *Ephedra distachya* (Shihata, Imaseki, & Yamazaki, 1957). James (1949) found slight increases in the alkaloid content of detached young leaves of *Atropa belladonna* supplied with sucrose and arginine or ornithine.

## H. The metabolic relations of alkaloids

The importance of alkaloids to the general metabolism of the plants that form them is far from clear, probably varying from one species to another. In many species alkaloid formation is associated with actively growing regions, perhaps even restricted to them. The elaborate patterns of alkaloid distribution in different organs and tissues also suggest some metabolic significance, but no clear indication of direct participation in metabolic processes can be given for most alkaloids. There is evidence that alkaloids are metabolized in the plant; transformation *in vivo* between different alkaloids are established, but little is known about their connexion with other metabolic processes.

Boussingault (1868), in a footnote to a brilliant exposition of the rôle of asparagine in plants, threw out the suggestion that in germinating potato sprouts it was replaced by solanine. Increased knowledge of the chemistry of solanine hardly indicates any metabolic similarity to asparagine, but it is an active metabolite and Boussingault was probably right in assuming that it had some function in the physiology of the potato. Baup (1826) pointed out that potato sprouts contained much more solanine than the tubers. Solanine has a large medical literature as it causes toxicity in potato sprouts, sometimes eaten through ignorance or stress of hunger, and in tubers becoming green after storage

in the light. The latter are the usual cause of outbreaks of poisoning; children have been poisoned by eating leaves, flowers, and unripe fruits of potato. The fatal dose for man is 200 to 400 mg of solanine. These alkaloids have been studied as insecticides and as starting points for the synthesis of steroid hormones.

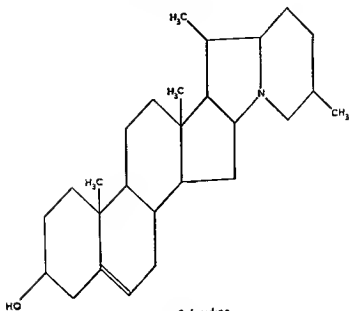
Zwenger & Kind (1861) found solanine to be a glycoside and named the alkaloidal aglycone solanidine. Soltys & Wallenfels (1936) established its structural relation to the sterols, thus confirming the prescient remark of Desfosses (1821) that solanine closely resembled cholesterol. The sterol skeleton occurs also in the veratrine group of alkaloids, found in several species of Liliaceae (*Veratrum album*, *V. viride*, *Schoenocaulon officinale*) (Craig & Jacobs, 1943*a, b*) and Apocynaceae (*Funtumia africana*, *F. latifolia*, *Holarrhena floribunda* (Janot, Cavé, & Goutarel, 1960; Janot, Qui, & Goutarel, 1960). Two less well-known alkaloids containing the sterol skeleton occur in *Calotropis procera* (Asclepiadaceae), the active ingredient in an African arrow poison. Each of these alkaloids has one atom of nitrogen and one of sulphur in the molecule, which probably contains a thiazoline ring (Hesse & Gampp, 1952; Hesse & Lettenbauer, 1957).

It is now known (Kuhn & Löw, 1955; Kuhn, Löw, & Trischmann, 1955) that the solanine of earlier workers is a mixture of glycosides. Six glycoalkaloids containing solanidine were obtained from *Solanum tuberosum*, and also from *S. chacoense*. Their constitutions are as follows:

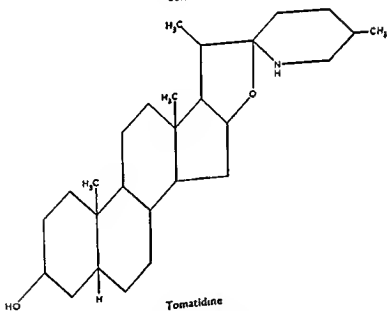
$\alpha$ -solanine:	solanidine-galactose-glucose-rhamnose
$\beta$ -solanine:	solanidine-galactose-glucose
$\gamma$ -solanine:	solanidine-galactose

$\alpha$ -chaconine:	solanidine-glucose-rhamnose-rhamnose
$\beta$ -chaconine:	solanidine-glucose-rhamnose
$\gamma$ -chaconine:	solanidine-glucose

Another triglycoside, solanidine-xylose-xylose-glucose (solacauline), occurs in *Solanum acaule* according to Schreier (1954); the hotanical identification of his material has, however, been queried (Petrochenko, 1957). The metabolism of individual glycoalkaloids in this series seems not to have been studied, though Paseshnikchenko & Guseva (1956) have published methods for their quantitative separation. *Solanum tuberosum* contains more chaconine than solanine (Paseshnikchenko, 1957). The enzymatic splitting of the glycoalkaloids into their aglycone



Solanidine



Tomatidine

FIG. 70.

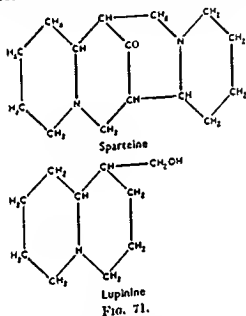
and carbohydrate constituents is very specific. Petrochenko (1953) found an enzyme in potato sprouts which split solanine but not tomatine or demissine; Prokoshev, Petrochenko, & Paseshnichenko (1956) obtained an extract from tomato leaves which split tomatine and demissine but not solanine. Species forming steroidal alkaloids (*Solanum tuberosum*, *S. aviculare*, *S. xanthocarpum*, *Lycopersicum pimpinelli-*

*folium*) also contain closely related steroidal sapogenins (Sato & Latham, 1953, Schreiber, 1957) These sapogenins also occur in genera, e.g. *Dioscorea*, not known to contain the corresponding alkaloids. Formation of the alkaloids is increased by the ultra violet part of the solar spectrum. Plants grown under glass, which absorbs much of this radiation, thus contain less steroidal alkaloid than those grown in field conditions (Sander, 1956, Schreiber, 1957), the content of the corresponding sapogenins is, however, higher under glass, suggesting that they may share a common precursor with the alkaloids. Solanidine and its reduction product demissidine, the aglycone from the glycoalkaloid of *Solanum demissum*, are secondary amines, tomatidine, the aglycone of tomatine (*Lycopersicum esculentum* and other species of *Lycopersicum*), has a large part of the same carbon skeleton, but differs in being a tertiary amine with a heterocyclic ring of four carbon atoms and one oxygen atom (Fig. 70). *Solanum aviculare* contains glycoalkaloids with aglycones structurally similar to tomatidine (Kulin & Löw, 1955).

Solanine, like many other alkaloids, is characteristic of metabolically active tissues. Green sprouts grown in the light contain more than etiolated sprouts grown in the dark, but even the latter have more than the tuber. Flower buds and young leaves contain much solanine, it is apparently metabolized in aging flowers and leaves (Votehal, 1889, Naumov, 1938, Arutyunyan, 1940, Lampitt, Bushill, Rooke, & Jackson, 1943, Wolf & Duggar, 1946). There is a marked increase in the solanine content of potato tubers that turn green through being kept in the light. This increase is particularly marked with young tubers (Griebel, 1924, Bömer & Mattis, 1924, Conner, 1937). The young potato plant has a high concentration of solanine, the concentration falls during the later stages of development, though the absolute amount per plant increases. In the aging plant the total content of solanine probably decreases. The concentration falls in the growing tuber by dilution rather than by an actual loss of alkaloid (Wolf & Duggar, 1946). Young fruits contain much solanine but it largely disappears during ripening and the seeds contain very little (Votehal 1889). This contrasts with the position in *Lupinus luteus* where during the ripening period the alkaloid content decreases considerably in the vegetative parts, concurrently with an increase in the seeds, to which alkaloid may be translocated (Sabalitschka & Jungermann, 1925). In *Sarothamnus scoparius* (broom), whose alkaloids resemble those of lupin, the vegetative parts and the young seeds contain sparteine, but in the ripe seeds the oxidized compounds lupinine (Fig. 71) and hydroxylupinine



predominate (Jaminet, 1954). In species of *Solanum* (Prokoshev, Petrochenko, Ilyin, Baranova, & Lebedeva, 1952) and of *Lycopersicum* (Sander, 1956) steroidal alkaloids pass from the leaves to the fruits, where to a large extent they are broken down. In tomato (*Lycopersicum esculentum*) the tomatine content of the whole plant increases considerably above the normal maximum if all the flowers are removed. Prevention of fruit formation eliminates the main site where the alkaloid is broken down (Sander, 1956). The glycoalkaloids are active metabolites, but their formation and breakdown in the plant remain obscure.



The formation of scopolamine from hyoscyamine in *Datura ferox* has been studied by Mothes & Romeiko (1955) and by Romeiko (1959). Shoots of this species contain mainly scopolamine and only traces of hyoscyamine, but the latter is formed in considerable amounts in the roots. Scions of *Cyphomandra betacea* on stocks of *Datura ferox* accumulate hyoscyamine, which can also be detected in the bleeding sap of decapitated plants of *D. ferox*. Leaves or shoots of *Datura ferox* grown as scions on *Cyphomandra betacea* as stock formed scopolamine from hyoscyamine supplied artificially. In *Datura ferox* hyoscyamine is thus formed in the roots and converted to scopolamine in the shoot.

### I. Alkaloids and their *N*-oxides

Auerbach & Wolfenstein (1901) prepared the *N*-oxide of nicotine by oxidizing it with hydrogen peroxide. They determined the structure

of the product, which was then without known analogues among natural products. Polonovski & Nitzberg (1915) were probably the first to isolate from natural sources the *N*-oxide of an alkaloid. They obtained from seeds of *Physostigma venenosum* an alkaloid which they named genescrine, and recognized as the *N*-oxide of eserine, long known from the same seeds. The chemistry of alkaloid *N*-oxides was discussed by Polonovski & Polonovski (1926); almost all the examples of this class of compound then known were synthetic, but many have since been isolated from natural sources. Species known to contain *N*-oxides of alkaloids are listed by Areshkina (1957a). They all belong to the families Boraginaceae, Compositae, and Leguminosae. The alkaloids found up to the present time as oxides in plants belong mostly to the pyrrolizidine series; eserine is an exception, its nucleus being formed by a benzene ring fused to two pyrrolidine rings. Oxides of 5-hydroxyindolylethyldimethylamine (bufotenine) and of *N,N*-dimethyltryptamine occur in *Piptadenia peregrina* together with the corresponding unoxidized compounds (Fish, Johnson, & Horning, 1955). Epilupinine, a quinobizine derivative, occurs mainly as *N*-oxide in seeds of *Lupinus varius* (Crow & Riggs, 1955).

*N*-oxides of alkaloids are also microbial products. *Pseudomonas pyocyanea* excretes into culture solution a substance antagonizing the antihacterial action of dihydrostreptomycin and containing (Cornforth & James, 1956) the *N*-oxides of 2-*n*-heptyl-, 2-*n*-nonyl- and 2-*n*-undecyl-4-hydroxyquinoline.

A high proportion of the total alkaloid may be present as *N*-oxide, especially with alkaloids of the pyrrolizidine group. Recognition of this fact has led to a considerable upward revision of the alkaloid content of some species, as the *N*-oxides are often missed by extraction procedures successful with reduced alkaloids. This is important in the assay of many weeds containing pyrrolizidine alkaloids, which are liver poisons causing serious losses of stock. Plants containing much alkaloid as *N*-oxide may be highly toxic though appearing almost alkaloid-free with the usual extraction methods. *N*-oxides of *Senecio* alkaloids are more palatable and hence more dangerous to stock than the corresponding reduced compounds (Schoental, 1955). Areshkina (1951, 1957b) showed that in *Senecio platyphyllus* the bulk (80 to 90 per cent) of the total alkaloid was *N*-oxide, the only exception was in the roots during the dormant period, when all the alkaloid was in the reduced form. When the plant, a perennial herb, returns to activity in the next growing season the alkaloid stored in the root in the reduced form is

promptly re-oxidized. In *Heliotropium europaeum* (Boraginaceae) (Culvenor, Drummond, & Price, 1954) and *Senecio quadridentatus* (Compositae; formerly placed in *Erechtites*) (Culvenor & Smith, 1955) a very high proportion of the total alkaloid is in the oxidized form. Koekemoer & Warren (1951) reported similar results for *Senecio adnatus*, *S. brachypodus*, *S. hygrophilus*, and *S. isatideus*; yields from these species were greatly increased by reduction before extraction with chloroform. In *Lupinus varius* (Crow & Michael, 1957) and *Crotalaria spectabilis* (Culvenor & Smith, 1957b) the same great excess of oxidized over reduced alkaloid is found in the seeds; in other parts of the plant there is a substantial amount of N-oxide, but more than half of the total alkaloid is reduced. Seeds of *Crotalaria retusa* in different samples show widely varying proportions (2 to 64 per cent) of the total alkaloid as N-oxide (Culvenor & Smith, 1957a). Storage of N-oxides in these seeds contrasts with storage as reduced alkaloid in the roots of *Senecio platyphyllus* (Areshkina, 1951); the seeds of this species, however, contain alkaloid mainly as N-oxide. Areshkina (1957b) showed that a homogenate of the rootstock of *S. platyphyllus* reduced alkaloid N-oxides when supplied with malic acid or ethyl alcohol as hydrogen donors; no reduction was observed with ascorbic acid. The relation of these reversible oxido-reductions to other metabolic processes is not yet clear. A similar reversible oxido-reduction probably occurs in hemlock (*Conium maculatum*) between  $\gamma$ -coniceine and conine (Fairhairn & Challen, 1959).

#### J. Alkaloids during the development of the plant

Changes in the alkaloid content of developing plants have been followed mainly with members of the Solanaceae forming alkaloids of the tropane (mydriatic) or nicotine groups. The results vary in detail but show a general similarity. The seeds are poor in alkaloid, which is formed by the seedling early in germination. The total alkaloid content of the plant increases steadily during the early stages of development, reaches a maximum about the time of flowering, and then decreases. The decrease may be in part due to losses by leaf-fall, but probably reflects a transformation of alkaloid to other substances, especially in ripening seeds. Individual leaves increase their alkaloid content up to the onset of senescence, when it decreases. This general picture appears in the results of many authors, e.g. Vlădescu (1938c) (*Nicotiana tabacum*), Guillon (1950) (*Datura stramonium*), and Shpilnenya (1953) (*Scopolia carniolica*). Areshkina (1951) followed the changes in alkaloid content

during the seasonal development of *Senecio platyphyllus*, a perennial herb with a dormant period. The total alkaloid content of the root decreased slightly during the active period, apparently by translocation to the shoot. The shoot showed a marked increase in alkaloid, considerably exceeding the decrease in the root. At the end of the vegetative period alkaloid returned to the perennial root system. The proportions of the two main alkaloids, platyphylline and seneciophylline, varied considerably in the course of development; seneciophylline formed 32 per cent of the total alkaloid in the root at the beginning of the vegetative season and 18 per cent near its end; in mature leaves and seeds it formed 12 per cent. Marked qualitative changes in alkaloid content occur in *Smirnovia turkestanica* (Leguminosae) (Ryabinin & Ilyina, 1951). In May the plant contained only smirnovine, but in August smirnovinine and sphaerophysine were found.

Changes in the content of individual alkaloids during development of various species of *Atropa*, *Datura*, and *Hyoscyamus* have been followed by Hegnauer (1951), Evans & Partridge (1953), Romeike (1953), and other authors. The results obtained showed a general similarity. Scopolamine, the dominant alkaloid in the young seedling, is soon overtaken by hyoscyamine, which forms 80 per cent or more of the total alkaloid in mature *Atropa belladonna*. In *Hyoscyamus niger* the seedling contains almost exclusively scopolamine, but later hyoscyamine predominates. *Datura innoxia* contains more scopolamine than hyoscyamine at all stages, although the hyoscyamine content increases with advancing age in this species also. In *Duboisia myoporoides* (Trautner, 1947) scopolamine is the main alkaloid in the seedling and in some races throughout the life of the plant, a perennial which can become a fair-sized tree. In other races, hyoscyamine appears in seedlings about six months old and soon becomes the main alkaloid. A somewhat different picture is found in *Datura ferox* (Evans & Partridge, 1953), where scopolamine is always the main alkaloid. In the seedling meteloidine forms 26 per cent of the total alkaloid; in the mature plant only 7 per cent of the total alkaloid is meteloidine.

#### K. Biosynthesis of alkaloids

Much ingenuity has been applied by chemists to the synthesis of alkaloids, particularly by synthetic sequences based on naturally occurring compounds and taking place *in vitro* in 'physiological' or 'zellmöglich' conditions (dilute aqueous solutions at room temperature and pH 5 to 7). Pictet & Court (1907) suggested amino-acids, derived

from protein breakdown, as the main precursors of alkaloids. This view, adopted by most subsequent students of the problem, is supported by the association of alkaloid formation with protein breakdown in seedlings of *Datura* (Sukhorukov & Borodulina, 1932) and of *Ricinus* (Weevers, 1933).

Plants form several simple heterocyclic nitrogenous compounds which seem potential precursors of alkaloids. Piperidine occurs in pepper (Johnstone, 1888; Spath & Englaender, 1935) and in tobacco (Spath & Zajic, 1936). Pictet & Court (1907) found *N*-methylpyrroline

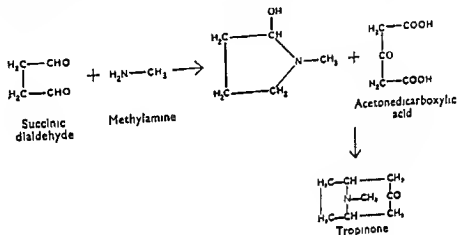


FIG. 72.

in pepper and pyrrolidine in tobacco and carrot. *N*-methylpyrrolidine is reported from tobacco (Späth & Biniecki, 1939) and *Atropa belladonna* (Goris & Larssonneau, 1921), and pyridine from coffee (Bertrand & Weisweiler, 1913). Most of these bases came from material subjected to processing (coffee, pepper, tobacco) or to chemical treatment (mother liquors from extraction of *Atropa*). This does not apply to the isolation of piperidine from *Petrosimonia monandra* and of *N*-methylpiperidine from *Girgensohnia diptera* (Yurashevski & Stepanov, 1939a, b). Both species belong to the Chenopodiaceae; the bases formed 1 per cent or more of the dry weight in the green parts. *Psilocaulon absimile*, known as a stock poison in South Africa, contains much piperidine (4.5 per cent of the dry weight); in the plant the base apparently exists, in part at least, as the hydrochloride (Rimington, 1934). Buehrer, Mason, & Crowder (1939) found pyridine as 2 per cent of the dry weight in *Aplopappus hartwegi* (Compositae). Cromwell (1943a) found *N*-methylpyrroline and *N*-methylpyrrolidine in *Atropa belladonna* and *Datura*

from ornithine by the methylating and oxidizing action of formaldehyde (Fig. 74), could condense with acetonedicarboxylic acid or acetoacetic acid to form hygrine and cuscohygrine (Fig. 75). These syntheses were realized in 'physiological conditions' by Anet, Hughes, & Ritchie (1949a) and by Galinovsky & Weiser (1950). Schöpf & Arnold (1945)

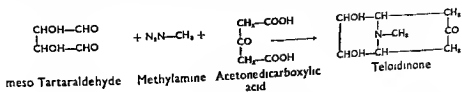


FIG. 76.

used mesotartaraldehyde to synthesize teloidinone (Fig. 76) by another reaction of the same type. Schöpf & Lehmann (1935) synthesized lobelanine (Fig. 77) on somewhat similar lines from glutaraldehyde, methylamine, and benzoylacetic acid. The synthetic product had the same configuration (meso) as the natural base. A compound (3-hydroxyl-3-phenylpropionic acid) closely related to benzoylacetic acid occurs in *Lobelia inflata* together with lobelanine (Wieland, Koschara, Dane, Renz, Schwartz, & Linde, 1939). Methylamine, used in several of these

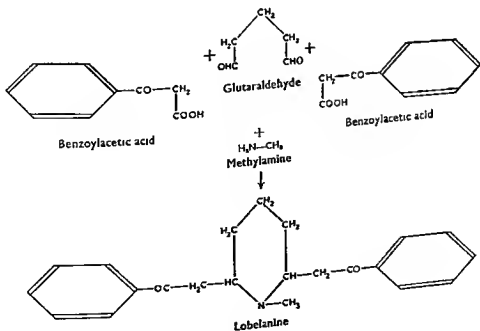
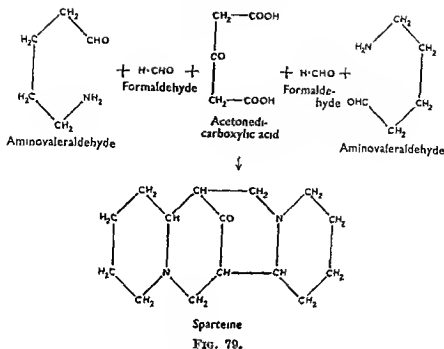
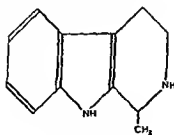


FIG. 77.

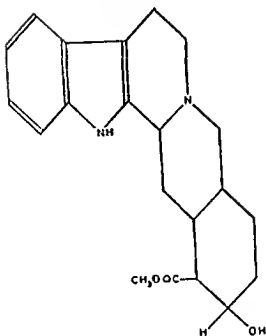


*racemosa* (Symplocaceae) and in *Peganum harmala* (Zygophyllaceae). Dihydroharman condenses with *o*-aminobenzaldehyde to a product oxidized by ferriocyanide at pH 7 to rutaecarpine, found in *Evodia rutaecarpa* (Schöpf & Steuer, 1945). Hahn & Werner (1935) and Hahn, Bärwald, Schales, & Werner (1935) also synthesized tetrahydroharman derivatives in very mild conditions and obtained from tryptamine and



*m*-hydroxyphenylpyruvic acid a base with the complex yohimbine skeleton (Fig. 81).

The possibilities of this approach, which concentrates upon condensation reactions between substances known or reasonably expected to occur in plant cells, have been discussed by several authors (e.g.



Yohimbine

FIG. 81.

Robinson, 1936, 1955; Schöpf, 1937; Hughes & Ritchie, 1952). Most of this work has been inspired by Robinson, who with an admirable combination of theory and experiment has over the last 40 years clarified many problems in the structure of alkaloids. The results of this approach cannot be more than suggestive for studies in biogenesis, as the simplest sequence leading *in vitro* to a particular compound need not necessarily represent its mode of formation *in vivo*. Robinson (1936) emphasized that 'all such schemes are regarded as too simple in details and are only advanced in broad outline'. Syntheses achieved *in vitro* in mild conditions do, however, provide valuable pointers for studies in the plant. The theoretical concepts evolved by Robinson and other workers in this field have been very valuable in suggesting fruitful approaches to structural and synthetic problems in the alkaloids. Brilliant examples, e.g. the work of Woodward (1948) on the structure of strychnine, and the proposal on theoretical grounds (Robinson, 1948) of a structure for emetine later confirmed by synthesis (Battersby & Openshaw, 1950), show the value of biosynthetic considerations in elucidating the structure of complex alkaloids, and in suggesting elegant and powerful approaches to their synthesis.

Some details of the chemical methods used in these studies require modification in applying their results to biosynthesis. Formaldehyde,



for instance, may not take part as such in methylations within the cell, where various equivalent one-carbon molecules or portions of molecules (e.g. methyl alcohol, formic acid, the terminal residues of glycine or serine) may replace it. Formation *in vivo* of the  $-\text{CH}_3$ ,  $-\text{OCH}_3$ , and  $-\text{NCH}_3$  groups so common in alkaloids may also be achieved by transmethylation from such compounds as betaine, methionine, dimethylthetin, dimethylpropiothetin, and methylethylthetin (Fig. 82). The thetins are at present known mainly from algae. Transmethylation from methionine is an efficient source of methyl groups in the biosynthesis of several alkaloids: ricinine (Dubeck & Kirkwood, 1952); bordenine

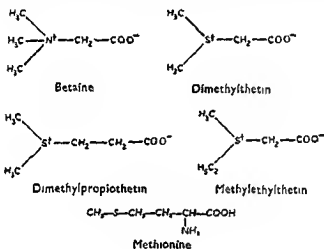


FIG. 82.

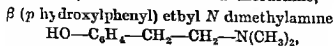
(Matchett, Marion, & Kirkwood, 1953); protopine (from *Dicentra*) (Sribney & Kirkwood, 1953); nicotine (Dewey, Bjerrum, & Ball, 1954); hyoscyamine (Marion & Thomas, 1955); codeine, morphine, and thebaine (Battersby & Harper, 1958b). Formate, formaldehyde, and glycine are also sources of methyl groups for some of these alkaloids, but are often less effective than methionine. Formaldehyde and the  $\alpha$ -carbon of glycine are, however, efficient precursors of the methyl group of nicotine (Byerrum, Ringler, Hamill, & Ball, 1955; Byerrum, Hamill, & Ball, 1954). Some mould fungi, notably *Penicillium brevicaulis* (*Scopul-ariopsis brevicaulis*), methylate inorganic arsenic, selenium, and tellurium to the gases trimethylarsine, dimethylselenide, and dimethyltelluride. Gosio (1897) showed that moulds produced a poisonous arsenical gas. The mechanism of its production, together with that of the analogous selenium and tellurium compounds, has since been studied;

here also methionine is a very effective methylating agent (Challenger & Higginbottom, 1935, Challenger, Lisk, & Dransfield, 1953)

Acetone and acetonedicarboxylic acid are widely used reagents for the synthesis of alkaloids *in vitro* in 'physiological conditions' The reactive dicarboxylic acid does not seem to be reported in plants Acetone itself occurs in some species as the cyanogenic glucoside phaseolunatin (linamaroside), which on enzymatic hydrolysis yields glucose, hydrocyanic acid, and acetone The glucoside occurs in flax (*Linum usitatissimum*) (Jorissen & Hairs, 1887), *Phaseolus lunatus* (Dunstan & Henry, 1903), manioc (*Manihot utilisima*) (Dunstan, Henry, & Auld, 1906), and in several species of *Dimorphotheca* (Compositae) (Rimington & Steyn, 1935) It is recorded also from a few other species of Leguminosae and Euphorbiaceae The higher homologue, lotusaustraloside, which on enzymatic hydrolysis gives methyl ethyl ketone, is found in *Lotus australis* and *Trifolium repens* (Innemann & Cooper, 1938) Aminoacetone, formed from threonine by *Staphylococcus aureus* (Elliott, 1959), would be a plausible precursor of alkaloids if it occurs in higher plants

The species known to form acetone are not prominent as producers of alkaloids In any case they accumulate little or no free acetone, storing it as glucoside It is thus unlikely that synthesis of alkaloids *in vivo* is based on acetone or its decarboxylic acid to the extent which studies *in vitro* might suggest The artificial syntheses may well, however, correspond in broad outline to those occurring naturally Acetone may be formed transiently and utilized without ever accumulating to a detectable level, or it may be replaced by simpler substances condensing to give its structural equivalent in the alkaloid molecule Parker, Raphael & Wilkinson (1959) introduced a new approach by synthesizing tropinone, pseudopelletierine and lobelanine from the acetylenic compounds hexa 1 5 diyne and hepta 1 6 diyne Numerous acetylenic compounds with one to several triple bonds are known as plant products, and may well be possible precursors of alkaloids Some alkaloids are synthesized by several routes in the laboratory, *in vivo* also a single compound may arise in different ways especially when it occurs in several unrelated species

The synthetic pathways leading *in vivo* to some of the simpler alkaloids (or as they may also be considered more complicated amines related to amino acids) are well established Hordenine,



is a good example of this group. It was isolated from grasses by Gaebel (1906) and Léger (1906). Spáth (1919), however, showed hordenine to be identical with anhaline, isolated by Heffter (1894) from the cactus *Anhalonium fissuratum*. It occurs in *Trichocereus candicans* and other cacti (Reti, 1933), and in the mistletoes *Phoradendron californicum*, *P. flavescens*, and *P. villosum* (Crawford & Watanabe, 1914, 1916).

Raoul (1937*a, b*) showed that barley seedlings synthesize hordenine and suggested that in the plant it arose from tyrosine via tyramine, a synthesis (Fig. 83) which he realized *in vitro* in physiological conditions. Tyramine is formed by bacterial decarboxylation of tyrosine (Ackermann, 1909; Barger & Walpole, 1909). A pyridoxal-dependent tyrosine decarboxylase occurs in *Streptococcus faecalis* (Epps, 1944). Such

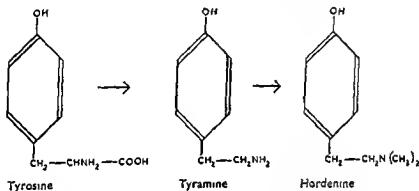
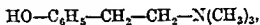


FIG. 83.

enzymes have not been isolated from higher plants, but indirect evidence suggests their existence. Tyrosine decreases as hordenine increases in barley seedlings (Raoul, 1937*b*). These seedlings contain tyrosine, tyramine, *N*-methyltyramine, and hordenine (Erspamer & Falconieri, 1952); *N*-methyltyramine may be more prominent than tyramine (Kirkwood & Marion, 1950). The related compounds hydroxytyramine and *N*-methylhydroxytyramine occur in broom (*Sarothamnus scoparius*) (Schmallfuss & Heider, 1931; Correale & Cortese, 1953). Correale & Cortese (1954) reported in macerated broom seedlings the enzymatic sequence: tyrosine  $\rightarrow$  tyramine  $\rightarrow$  hydroxytyramine. Yields were low at each stage. Tyramine occurs in *Phoradendron* (Loranthaceae) (Crawford & Watanabe, 1914, 1916) and in a few other species, generally as a minor constituent. Fowden & Done (1954), however, found it to contain 90 per cent of the amino nitrogen in exudates from cut flower-stalks of *Crinum yuccaeiflorum* (Amaryllidaceae). In roots, bulbs, and

leaves of this species it appeared in smaller amounts than other amino-acids and amides.

The biosynthesis of hordenine has been further studied by a Canadian group. Barley seedlings supplied with labelled tyramine (Leete, Kirkwood, & Marion, 1952) or tyrosine (Leete & Marion, 1953a) formed *N*-methyltyramine and hordenine labelled in the corresponding positions. No labelled tyramine was recovered, suggesting rapid utilization in the plant. Methionine was an effective methyl donor (Leete & Marion, 1954) in these reactions. Massicot & Marion (1957) demonstrated in barley seedlings the sequence: phenylalanine  $\rightarrow$  tyrosine  $\rightarrow$  tyramine  $\rightarrow$  *N*-methyltyramine  $\rightarrow$  hordenine. The presence (Ersparmer & Falconieri, 1952) of a quaternary ammonium base in barley seedlings suggests that hordenine may be methylated to  $\beta$ -(*p*-hydroxyphenyl)-ethyltrimethylammonium (candicine):



which occurs together with hordenine in *Trichocereus lamprochorus* and other cacti (Reti, 1933). Similar sequences starting with di- or trihydroxyphenylalanine would lead respectively to coryncine, the dihydroxy analogue of candicine, and to mescaline, the trimethoxy analogue of tyramine. These compounds are both found in cacti, mescaline from *Anhalonium fissuratum* being well known as producing fantastic highly coloured visions in man. Leete (1959) supplied tyrosine- $\alpha\text{-C}^{14}$  to a cactus (*Anhalonium lewinii*, probably synonymous with *A. fissuratum*). Mescaline with radioactive carbon in the corresponding position was formed, indicating tyrosine as a direct precursor of the alkaloid. James & Butt (1957) showed that barley roots developed from isolated embryos contained no hordenine, but synthesized it if supplied with an extract of barley endosperm. This extract contained no tyramine, *N*-methyltyramine, or hordenine. Tyramine and *N*-methyltyramine were metabolized to hordenine if methionine was supplied at the same time; otherwise no synthesis occurred.

Gramine (indolyl-(3)-methyl-*N*-dimethylamine), another simple alkaloid found in grasses (barley Von Euler & Hellström, 1933; *Arundo donax*: Orekhov & Norkina, 1935), is closely connected metabolically with tryptophan. Bowden & Marion (1951) supplied tryptophan- $\beta\text{-C}^{14}$  to barley plants and isolated gramine labelled in the same position. Leete & Marion (1953b) showed that tryptophan labelled with  $\text{C}^{14}$  both in the  $\beta$  position and in the methylene group gave gramine without change in the carbon skeleton of the molecule (Fig. 84). Intermediate

stages in the process, during which a carbon atom is lost from the side-chain, are not known. Tryptamine, the decarboxylation product of tryptophan, occurs in *Acacia floribunda*, *A. longifolia*, and *A. pruinosa* (White, 1944). Closely related compounds include dipterine (*N*-methyltryptamine) from *Girgensohnia diptera* (Cbenopodiaceae) (Yurashevski & Stepanov, 1939b) and its 5-methoxy derivative in the grass *Phalaris arundinacea* (Wilkinson, 1958c); 5-hydroxytryptamine, found in the hairs covering the pod of *Mucuna pruriens* and possibly responsible for the intense itch which they cause (Bowden, Brown, & Batty, 1954); bufotenine, already mentioned as a substance produced by animals, plants, and fungi; and *N,N*-dimethyltryptamine isolated from leaves of *Prestonia amazonica* (Apocynaceae) (Hochstein & Paradies, 1957), and from seeds of *Piptadenia peregrina* (Fish, Johnson, & Horning, 1955).

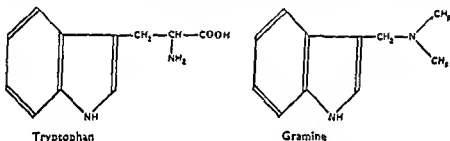


FIG. 84.

5-Hydroxytryptamine has been found in *Gossypium hirsutum* (Malvaceae) and *Symplocarpus foetidus* (Araceae) by Bulard & Léopold (1958). It occurs in appreciable amounts (about 8 mg/fruit, evenly divided between pulp and peel) in the banana fruit (*Musa sapientum*, Musaceae) (Waalkes, Sjoerdsma, Creveling, Weissbach, & Udenfriend, 1958; Cartier, Moreau, & Geoffroy, 1958) and in pineapple (Bruce 1960). It has marked effects on the human body when injected but is much less active when taken by mouth; its presence is therefore unlikely to lead to physiological disturbance even in persons eating large amounts of bananas. Bananas or pineapples in the diet can, however, upset clinical biochemical tests based on the presence of this substance (called serotonin in animal biochemistry) in the urine, where much of it is excreted when they are eaten. Serotonin is stated to be as active as  $\beta$ -indoleacetic acid in the oat coleoptile test for auxins (Niaussat, Laborit, Dubois, & Niaussat, 1958).

The hallucinations produced in man by mescaline (3,4,5-trimethoxyphenylethylamine) and their application in religious rites by certain

peoples of Mexico have been extensively studied. A somewhat similar use of preparations from *Piptadenia peregrina* which contain bufotenine (5 hydroxyindolyl ethyldimethylamine) has been reported from Haiti (Stromberg, 1954). It has been shown that hallucinogenic mushrooms (*Psilocybe aztecorum*, *P. caerulescens* var *mazatecorum*, *P. mexicana*, *P. semperiana*, *P. zapotecorum*, *Stropharia cubensis*) used ritually in Mexico contain two compounds with a general structural resemblance to mescaline and bufotenine. These are psilocin (4 hydroxydimethyl tryptamine) and psilocybin, in which the hydroxyl group of psilocin is phosphorylated (Heim, 1956, Heim & Hofmann, 1958, Hofmann, Heim, Brack, & Kohel, 1958, Hofmann & Troxler, 1959).

Much experimental work on the biosynthesis of pyrrolidine and piperidine alkaloids (e.g. hygrine, lobeline, nicotine, anabasine) has been inspired by theoretical suggestions (Winterstein & Trier, 1910, Robinson, 1917b) of ornithine and lysine as the respective precursors of pyrrolidine and pyridine rings. Klein & Linser (1933b) reported that detached tobacco shoots placed in solutions of proline, ornithine, or glutamic acid contained more nicotine than control shoots placed in culture solution containing mineral salts only. They interpreted their results as showing a synthesis of nicotine from the amino acids supplied. Gorter (1936) was unable to confirm the observations of Klein & Linser (1933b). He found that after 14 days the plants supplied with amino acids had more nicotine than the controls but both had less nicotine than at the start of the experiment. The data of both Gorter and Klein & Linser are difficult to assess, owing to sampling difficulties and the rather small changes observed in nicotine content. The use of shoots may also have confused the issue, as it is now known that nicotine is formed mainly in the root system. Later work (Dewey, Byerrum, & Ball, 1955, Leete, 1955, Leete & Siegfried, 1957) using ornithine labelled with  $C^{14}$  in the  $\alpha$  position, has shown that it is indeed a precursor of nicotine in tobacco, radioactivity from ornithine appeared in carbon atoms 2 and 5 of the pyrrolidine ring, showing that ornithine is not incorporated directly into the nicotine molecule. Glutamic acid is an effective precursor for the pyrrolidine ring of nicotine, probably via ornithine (Lamberts & Byerrum, 1958).

No evidence has been found for a similar production of the pyridine ring of nicotine from lysine. Bothner By, Dawson, & Christman (1956) supplied sterile isolated roots of *Nicotiana tabacum* with lysine labelled either with  $N^{15}$  or with  $C^{14}$  in all positions. Little of the labelled nitrogen or carbon appeared in the nicotine formed, and that little was

mainly in the pyrrolidine, not the pyridine ring. Leete (1956) found that lysine-2- $C^{14}$  supplied through the roots was used in the synthesis of anabasine in *Nicotiana glauca*, all the labelled carbon appearing in the  $\alpha$  position in the piperidine ring. He found no utilization of lysine-2- $C^{14}$  for nicotine synthesis in *N. tabacum*. Grimshaw & Marion (1958) also found lysine unable to take part in the formation of the pyridine ring of nicotine. Results with anthranilic acid, another suggested precursor of this ring, were also negative. Bogdashovskaya (1954) reported that lysine was used in the formation of ricinine, which also contains a pyridine ring, in *Ricinus communis*; Grimshaw & Marion (1958), however, cite evidence against this. They suggest that the pyridine ring may be built up from small units arising from glycine or alanine, or alternatively from ammonia and non-nitrogenous precursors. Tamir & Ginsburg (1959) found that seedlings of *Ricinus communis* incorporated  $C^{14}$ -labelled lysine and  $\alpha$ -aminoadipic acid into ricinine, suggesting that they are used in its biosynthesis. Carbon from labelled acetate, propionate, and glycerol supplied to seedlings of *Nicotiana rustica* appeared in both rings of nicotine (Griffith, Hellman, & Byerrum, 1960). These authors proposed that the pyrrolidine ring arose from simple precursors via glutamic acid and the pyridine ring via  $\beta$ -alanine. The metabolic events leading to formation of the pyridine ring remain somewhat obscure, but lysine seems to be a precursor in ricinine at least.

Lysine uniformly labelled with  $C^{14}$  appears (Schiedt & Höss, 1958) to be a precursor of coniine in *Conium maculatum*, as suggested by Robinson (1955). Nicotinic acid is a precursor of the pyridine ring of nicotine in tobacco roots. Sterile tobacco roots fed with nicotinic acid labelled in the ring with  $H^3$  or  $C^{14}$  formed nicotine with the radioactive atoms in the pyridine ring (Dawson, Christman, & D'Adamo, 1956). A largely increased nicotine synthesis in tobacco seedlings supplied with nicotinic acid or nicotinamide was reported earlier by Pratesi, Ciferri, & Cambieri (1946); pyridine tartrate also increased nicotine synthesis (Ciferri, 1946). Nicotinic acid labelled in the carboxyl group with  $C^{14}$  led to labelled ricinine in *Ricinus communis* (Leete & Leitz, 1957).

Nicotinic acid labelled with tritium in the 2, 4, or 5 position led directly to nicotine in sterile cultures of excised tomato roots. Tritium was lost from nicotinic acid labelled in the 6 position, suggesting the occurrence of an intermediate of the 6-pyridone type between nicotinic acid and nicotine (Dawson, Christman, D'Adamo, Solt, & Wolf, 1958).

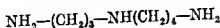
Nicotinic acid, an essential constituent of important co enzymes, probably occurs in all plants. In some fungi a long chain of intermediates leads from tryptophan to nicotinic acid via several derivatives of anthranilic acid. Many of these intermediates are formed in animals also, though the fact that nicotinic acid is a dietary essential for man and other animals suggests that in them the sequence does not include its formation. Tryptophan breakdown in higher plants may follow a different pathway. Leete, Marion, & Spenser (1955b) found that feeding tryptophan  $3\text{-C}^{14}$  led to no inclusion of  $\text{C}^{14}$  in trigonelline (the betaine of nicotinic acid) in peas, or in damascenine (closely related to 3-hydroxyanthranilic acid, a widespread metabolite of tryptophan in other organisms) in *Nigella damascena*. This evidence does not exclude completely the formation of nicotinic acid or anthranilic acid derivatives from tryptophan in higher plants, but suggests that at least in some species it is unlikely. Trigonelline and damascenine are characteristic products of the species in which their synthesis was sought, and it seems reasonable to suppose that they were being formed in the experimental plants. Aronoff (1956a, b) found no evidence of trigonelline formation in detached soybean shoots from 3-hydroxyanthranilic acid in which the carboxyl carbon was labelled with  $\text{C}^{14}$ .

Ornithine has been considered a likely precursor for the tropane alkaloids. Cromwell (1943b) suggested a scheme for the biosynthesis of tropinone and nortropinone (which by reduction and esterification with tropic ( $\alpha$ -hydroxymethylphenylacetic) acid would give respectively hyoscyamine and norhyoscyamine) from ornithine via putrescine. He found an enzyme in *Atropa belladonna* oxidizing putrescine (1,4-diaminobutane) to an aldehyde and ammonia, and showed putrescine to occur in *A. belladonna* and in *Datura stramonium*, it was recorded in the former species by Goris & Larssonneau (1921) and in the latter, rather doubtfully, by Ciameian & Ravenna (1911), it has also been reported in citrus juice (Hiwatari 1927, Herbst & Snell, 1948) and in potassium-deficient barley (Coleman & Richards, 1956). Putrescine is an essential growth factor for the bacteria *Hemophilus parainfluenzae* and *Neisseria perflava* (Herbst & Snell 1948, Martin Pelczar, & Hansen 1952) and for a mutant induced by ultra violet irradiation in the mould *Aspergillus nidulans* (Sneath 1955). Tetramethylputrescine occurs in *Hyoscyamus muticus* (Willstätter & Heubner 1907) and in *H. reticulatus* where it represents 1 per cent of the dry weight of roots from Central Asia (Konovalova & Magidson 1928).

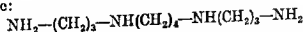
Tabor Rosenthal & Tabor (1958) studied the biosynthesis from



putrescine and methionine of the more complex straight-chain amines spermidine:



and spermine:



in the micro-organisms *Aspergillus nidulans*, *Azotobacter chroococcum*, *A. vinelandii*, *Escherichia coli*, and *Saccharomyces cerevisiae*. The reaction sequence for spermidine was formulated as follows:

- $$\begin{aligned} & \text{Mg}^{++} \\ (1) \text{ ATP} + \text{methionine} & \longrightarrow S\text{-adenosylmethionine,} \\ (2) S\text{-adenosylmethionine} & \rightarrow \text{CO}_2 + S\text{-adenosyl (5')-3-methyl-} \\ & \text{mercaptopropylamine,} \\ (3) S\text{-adenosyl (5')-3-methylmercaptopropylamine} & + \text{putrescine} \rightarrow \\ & \text{spermidine} + \text{thiomethyladenine.} \end{aligned}$$

Lunarino, an alkaloid from *Lunaria biennis*, yields spermidine on acid or alkaline degradation (Potier, Le Men, Janot, & Bladon, 1960). If these or similar amines are also formed in higher plants they would seem to be possible precursors of alkaloids, probably after molecular scission, as few alkaloids have three or four nitrogen atoms.

Cromwell (1943b) suggested that putrescine gave rise both to succindialdehyde and to an amino-aldehyde. The former condensing with methylamino and acetone would lead to tropinone; the latter, first cyclizing to a five-membered heterocyclic ring, would condense with acetone to give nortropinone. One detail of Cromwell's scheme, the occurrence of an unsaturated intermediate with a double bond in the position corresponding to C<sub>6</sub>-C<sub>7</sub> of the tropane ring, appears to be supported by a later *in vitro* synthesis of ecopolamine via a similar unsaturated compound (Fodor, Tóth, Koczor, Dobo, & Vincze, 1956). Cromwell (1943a) obtained increases in alkaloid content on injection of putrescine, together with glucose, into plants of *Atropa belladonna*. James (1946b), using the same species, concluded from feeding experiments that the nitrogen of the tropane alkaloids comes from the  $\gamma$ -amino group of the arginine-ornithine group of amino-acids and that the ring nitrogen of proline and  $\alpha$ -amino-nitrogen are not available.

Subsequent work with labelled compounds has confused rather than clarified the question of the precursors of tropane alkaloids. Diaper, Kirkwood, & Marion (1951) found that putrescine-1,4-C<sup>14</sup> supplied to *Datura stramonium* was taken up without formation of labelled hyoscyamine. Leete, Marion, & Spenser (1954), using the same species, found

that supply of ornithine labelled with  $C^{14}$  in the  $\alpha$  position gave hyoscyamine labelled in the two carbon atoms of the C—N—C 'bridge' ( $C_1$  and  $C_2$  of the tropane ring). The scopolamine present was completely inactive, a result interpreted to mean that ornithine was a precursor of hyoscyamine but not of scopolamine. This is unlikely in view of the close relationship between the two alkaloids. An alternative explanation is that scopolamine synthesis had ceased in the experimental plants; as already mentioned, production of scopolamine is characteristic of the early stages of development in most of the Solanaceae that produce tropane alkaloids.

Reinouts van Haga (1954, 1956, 1957), using isolated roots of *Atropa belladonna* in sterile culture, found that supply of ornithine and putrescine led both to increased growth of the roots and to higher concentrations of scopolamine as well as hyoscyamine. He also made the interesting observation that the first alkaloid to be formed in very young seedlings, before the appearance of scopolamine, was cuscohygrine, previously known only from *Erythroxylon coca* (Erythroxylaceae). This base was present in the roots of several mydriatic Solanaceae (*Atropa belladonna*, *Datura ferox*, *D. innoxia*, *D. metel*, *D. stramonium*, *Mandragora officinalis*, *Physochlaina orientalis*, *P. physaloides*, *Scopolia lurida*, and *S. sinensis*). Cuscohygrine (Fig. 74) contains two pyrrolidine rings joined by a bridge of three carbon atoms. The author suggests that it is a precursor of the tropane alkaloids, with which it shows, as noted by Willstätter (1900) and Robinson (1917b), a structural affinity. Its formation is increased by supply of ornithine, possibly converted to proline which would be the direct precursor. Cuscohygrine accumulates in *Atropa* scions, free of tropane alkaloids, on tomato stocks. The *Atropa* shoot may thus form the base without roots, but its possible production by tomato seems not to have been checked. Leete (1960a) supplied phenylalanine-3- $C^{14}$  to plants of *Datura stramonium*. They formed radioactive hyoscyamine and hyoscyne, all the activity being in the non-nitrogenous tropic acid portion of the alkaloid molecules.

Several workers have studied the biosynthesis of the ergot alkaloids. The ergot fungus (*Claviceps purpurea*) appears suitable for such work, as it grows in saprophytic culture, though it occurs naturally as a parasite of grasses. Early results with *Claviceps* in culture were disappointing. The indolyl residue of the ergot alkaloids suggests indole or tryptophan as likely precursors. De Tempe (1945) found no increase in alkaloid formation on adding indole, skatole, or tryptophan to cultures of *Claviceps*; Tyler & Schwarting (1954) also reported negative results

with tryptophan. Gröger, Wendt, Mothes, & Weygand (1959), however, obtained active incorporation of tryptophan- $\beta$ -C<sup>14</sup> into alkaloids formed by *Claviceps* in saprophytic culture, as did Taber & Vining (1959). In their experiments C<sup>14</sup>-labelled tryptophan led to substantial and essentially equal radioactivity in ergometrine, ergocornine, ergotamine, ergosine, ergosine, ergocryptine, ergocryptine, agroclavine, and elymoclavine, which thus probably arise by a common biosynthetic pathway. Radioactive alkaloids were also obtained from ergot growing parasitically, following injection of tryptophan- $\beta$ -C<sup>14</sup> into the stem of the host plant (rye) (Mothes, Weygand, Gröger, & Grisebach, 1958), slight synthesis contrasting with the negative results of Suhadolnik, Henderson, Hanson & Loo (1958). Labelled tryptophan leads to ergosine in saprophytic ergot cultures (Baxter, Kandel, & Okany, 1960), and to the indolic portion of the complex alkaloid ajmaline in *Rauwolfia serpentina* (Apocynaceae) (Leete, 1960b).

Gusova & Paseshnichenko (1958) showed that labelled acetate is used in the synthesis of solanine in the potato. In the dark, labelled carbon appeared both in the carbohydrate and the aglycone parts of the molecule; in the light, when sugars were presumably adequately supplied by photosynthesis, it appeared almost exclusively in the aglycone (solanidine). Battersby & Harper (1958a) and Leete (1958c) found tyrosine- $\alpha$ -C<sup>14</sup> to be a precursor of morphine in *Papaver somniferum*, as suggested by Robinson (1955). Kleinschmidt & Mothes (1959) showed that tyrosine uniformly labelled with C<sup>14</sup> was utilized in synthesis of morphine alkaloids by isolated leaves, isolated unripe capsules, and latex of *P. somniferum*. Both the isoquinoline and benzyl rings arose directly from the phenolic ring of tyrosine. Trier (1912) pointed out that benzylisoquinoline bases such as papaverine and laudanone could be derived from the aromatic amino-acids phenylalanine and tyrosine, via reactive derivatives such as amines and aldehydes. Syntheses on these lines were realized in 'physiological conditions' by Schöpf & Salzer (1940). Beal & Ramstad (1960) found pheoylalanine-2-C<sup>14</sup> to be a precursor of the isoquinoline alkaloid berberine in isolated shoots of *Berberis vulgaris*. Studies *in vitro* suggest that calycotomine, an isoquinoline alkaloid found in several Leguminosae, arises from 3,4-dihydroxyphenylalanine (Chatterjee & Chaudhury, 1960). The aromatic amino-acids arise in the plant from carbohydrate via shikimic acid and prephenic acids; these non-nitrogenous acids or their precursors may be incorporated into the carbon skeletons of alkaloids, whose high C/N ratio suggests that they are formed only in part from amino-acids.

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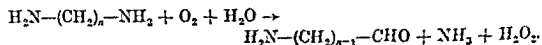
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Cromwell (1943*b*) found in *Atropa belladonna* an enzyme oxidizing putrescine. Later work has shown diamine oxidases to be widespread in plants, including non-alkaloidal species, and also in animal tissues. The general reaction for these enzymes (Tabor, 1951) may be written:



Enzymes of this type occur in species of several dicotyledonous families; they were found in all Labiatae tested and in several Leguminosae. In other families the enzymes appear sporadically; they were not found in the monocotyledons or gymnosperms examined. Although not detected in resting seeds of *Trifolium pratense* and *T. incarnatum*, they were active in early stages of germination (Werle & Raub, 1948; Werle & Zabel, 1948; Werle & Von Pechmann, 1949). These enzymes also deaminate histamine. They are stated to require two co-enzymes, riboflavin and pyridoxal. The animal enzymes require pyridoxal only, according to Sinclair (1952) and Davison (1956); Goryachenkova (1956) reported requirements for pyridoxal and flavin adenine dinucleotide in enzymes from animal tissues and from leguminous seedlings.

Diamine oxidase from pea seedlings oxidizes putrescine and its higher homologue cadaverine (1,5-diaminopentane), forming respec-

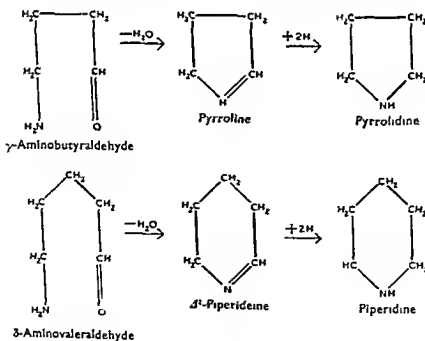
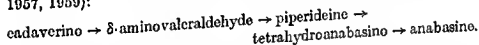


FIG. 85.

tively  $\gamma$ -aminobutyraldehyde and  $\delta$ -aminovaleraldehyde (Hasse & Maisack, 1955; Mann & Smithies, 1955). These amino-aldehydes cyclize readily, leading to pyrroline and  $\Delta^1$ -piperidine, which can be reduced to pyrrolidine and piperidine (Fig. 85). These ring compounds, arising from spontaneous cyclization of the products of a non-specific enzyme, are favourable starting points for alkaloid biosynthesis, as was strikingly demonstrated by the enzymatic formation (Hasse & Berg, 1957; Mothes, Schütte, Simon, & Weygand, 1959) of anabasine from cadaverine by extracts of pea seedlings. It is remarkable that the first enzymatic synthesis of an alkaloid *in vitro* was thus achieved by preparations from a non-alkaloidal plant. Mothes *et al.* (1959) showed, using cadaverine labelled in the C<sub>1</sub> and C<sub>6</sub> positions, that in the enzymatic synthesis the diamino is involved in the formation of both the piperidino and pyridine rings of anabasine. This is a surprising result, as in *Nicotiana glauca* the piperidine but not the pyridine ring of anabasine is formed from cadaverine (Leete, 1958a). The production of anabasine from cadaverine is formulated as follows (Hasse & Berg, 1957, 1959):



No spontaneous formation of anabasine was observed after lengthy autoxidation of cadaverine. Clarke & Mann (1959) isolated norhygrine and isopelletierine from reaction mixtures in which putrescine and cadaverine were oxidized in the presence of acetoacetate by enzymes from pea seedlings. Further alkaloids might arise through condensation of other  $\beta$ -keto compounds with unsaturated ring compounds produced by diamine oxidase. The occurrence of putrescine in some alkaloidal species is well established; the only records of cadaverine in higher plant materials not affected by bacterial decomposition seem to be in potato tubers (Yoshimura, 1934) and together with putrescine in old leaves and roots of pea plants (Miettinen, 1955). The pea plant thus contains both putrescine and cadaverine, together with an enzyme oxidizing them to products that *in vitro* readily cyclize to alkaloids; the plant, however, does not form these alkaloids in detectable amounts. The apparent potential of this species for alkaloid synthesis thus contrasts strongly with its actual performance; this suggests caution in applying to the intact plant the results of model experiments with enzymes, even when they act on naturally occurring substrates. Possibly the pea may be regarded as a species that has acquired the ability to form

products metabolically more useful than alkaloids from the diamines. Lysine and cadaverine are precursors *in vivo* of the quinolizine alkaloids lupinine and sparteine in *Lupinus luteus* (Schutte & Nowacki, 1959). The diamines are formed (Ellinger, 1900) by the bacterial decarboxylation of lysine to cadaverine and of ornithine to putrescine. Enzymes catalysing these reactions have not, however, been found in higher plants, where the diamines may be formed by some other pathway.

The formation of alkaloids in the plant often involves remarkably specific esterifications. In the mydriatic Solanaceae scopolamine is esterified by tropic acid, hydroxytropine by isovaleric acid,  $\psi$ -tropine by tiglic (*cis*-1,2-dimethylacrylic), and nortropine by  $\alpha$ -methylglutytic or  $\beta$ -methylbutyric acid (Trautner, 1947; Barger, Martin, & Mitchell, 1938). In *Coniolum pseudocantabricum* tropine and nortropine are esterified with veratric (3,4-dimethoxybenzoic) acid (Orekhov & Konovalova, 1934, 1935), and in *Erythroxylon coca*  $\psi$ -tropine with benzoic acid (Karrer, 1938). Other esterifying acids include methyl-ethylglycolic and acetic in *Veratrum* (Kramer & Acheson, 1946), sulphoacetic in *Erythrina* (Folkers, Koniuszy, & Shavel, 1944), and nitric in *Bursera madagascariensis* (Resplandy, 1957). The numerous pyrrolizidine alkaloids isolated from *Senecio* and some species of Boraginaceae and Leguminosae are formed by the combination of a comparatively small number of bases united with an almost bewildering variety of 'necic' acids (Leonard, 1950; Kuffner, 1957). The complexity and specificity of esterification in the plant raise difficult biochemical problems. Trautner (1947) pointed out that acids esterifying tropane bases in the Solanaceae are formally related to isoprene, thus bringing together two particularly complex groups of plant products, the alkaloids and the terpenes.

## L. Biological breakdown of alkaloids

Advances in our knowledge of alkaloid biosynthesis have had little counterpart in the field of their breakdown. There is evidence that this occurs in several plants, but little is known about the pathways involved or the products formed.

Heckel (1890) showed that, in several species with alkaloid-rich seeds (*Sterculia acuminata*, *Strychnos nux-tomica*, and *Physostigma tenosum*), alkaloids disappeared during germination and were apparently utilized in the seedling after conversion to other substances. Nicotine disappears in detached tobacco leaves (Smirnov & Izvosnikov, 1930; Vickery Pucher, Wakeman, & Leavenworth, 1933) Chaze (1931)



and Tsujita, Nawa, & Sakaguchi (1959) found measurable losses of nicotine by volatilization from tobacco leaves. The losses can account for only a small part of the alkaloid disappearing in detached leaves. At the acidity of tobacco leaf-sap (about pH 5.5) less than 1 per cent of nicotine occurs (Vickery & Pucher, 1929) as the free base, i.e. in a volatile form. Dawson (1940) reported nicotine to be metabolized in excised tobacco shoots; enzymes breaking down nicotine are recorded from tobacco leaves (Fodor & Reifenberg, 1927; Enders & Glawe, 1942). Mashkovtsev, Tsapkova, & Mniseyeva (1954) and Mashkovtsev & Sirotenko (1956) found that starved roots and shoots of tobacco broke down both endogenous and added nicotine; 70 to 80 per cent of the nitrogen of nicotine broken down in the roots appeared as ammonia. Tso & Jeffrey (1959) supplied  $N^{15}$ -labelled anabasine, nicotine, and normicotine via the roots to plants of *Nicotiana rustica* grown in water culture. Similar experiments were made with *N. glauca* using nicotine doubly labelled with  $C^{14}$  and  $N^{15}$ . The alkaloids supplied were metabolized by the plants; some of the labelled carbon and nitrogen appeared in other alkaloids, but the larger part was found in insoluble organic substances.

Schröter (1957) infiltrated  $C^{14}$ -labelled nicotine into detached leaves and shoots of *Nicotiana glauca*, where it formed anabasine and to a lesser extent normicotine. Leete & Bell (1959) found that intact plants of *Nicotiana tabacum* metabolized labelled nicotine actively in the roots but only sluggishly in the leaves. Nicotine acted as a methyl donor in the synthesis of choline. Ilyin (1959) also reported demethylation of nicotine and utilization of its methyl groups in *N. tabacum*. Bose, De, & Mohammad (1956) obtained from *N. glauca* and *N. tabacum* crude enzymatic preparations catalysing the demethylation of nicotine to normicotine, the eliminated methyl group being transferred to ethanolamine. The methylating appeared to be specific, the enzyme failing to methylate normicotine or guanidoacetic acid. Tropano alkaloids break down in aging leaves of *Datura inermis* (Shpilevaya, 1959); the breakdown begins earlier and is greater, relative to the initial content, than that of chlorophyll. The glycoalkaloids of potato and tomato are split by very specific enzymes present in the leaves, but the process has been studied only in the initial stage where sugars and steroidal aglycones are formed from the glycosides (Petrochenko, 1953; Prokoshev, Petrochenko, & Paseshnikhenko, 1956).

Some information is available on the early products of nicotine conversion during fermentation of tobacco leaves. Various 3-substituted

pyridines are formed, including 3-pyridylmethylketone and 2,3-dipyridyl (Frankenburg, Gottscho, Mayaud, & Tso, 1952). An earlier product is cotinine, a major component (Frankenburg & Vaitekunas, 1957) of the bases formed from nicotine in fermented cigar leaf. It differs from nicotine only in the presence of an oxygen atom, which converts the pyrrolidine to a pyrrolidone ring. Cotinine is also known as an autoxidation product of nicotine, and as a metabolite of nicotine in the dog (McKennis, Turnbull, & Bowman, 1958). Bueherer & Enders (1942) showed that some bacteria can break down nicotine to ammonia. Wada & Yamasaki (1954) isolated from soil a *Pseudomonas* using nicotine as a source of carbon and nitrogen. Two oxidation products were identified, 3-nicotinoylpropionic acid and pseudohydroxynicotine

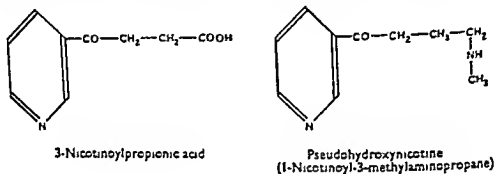


FIG. 86.

(1-nicotinoyl-3-methylaminopropane) (Fig. 86). (1)-6-Hydroxynicotine has been identified as the first product of oxidation of nicotine by *Pseudomonas fluorescens* (Hughes, 1952) and by an un-named soil bacterium (Hochstein & Rittenberg, 1959).

Wada, Kasaki, & Saito (1959) detected ammonia, cotinine, methylamine, myosmine, nicotinic acid, nicotyrine, and oxynicotine among the oxidation products of nicotine aerated at 30°C. Nonenzymatic reactions at moderate temperatures can thus effect considerable changes in the nicotine molecule. Hylin (1959) studied the breakdown of nicotine by *Achromobacter nicotinophagum*, a species isolated from tobacco seeds. In rapidly growing cultures nicotine was degraded via 6-hydroxynicotino to aliphatic products. Resting cells converted nicotine by successive oxidations to pseudohydroxynicotine, 3-succinoylpyridine, and 6-hydroxy-3-succinoylpyridine, which was not further metabolized. The organism did not attack tobacco alkaloids other than nicotine. Niemier, Bucherer, & Kohler (1960) isolated *Corynebacterium belladonnae*

from soil under plants of *Atropa belladonna*. It used atropine, hyoscyamine, and scopolamine as sole sources of carbon and nitrogen. Tropic acid, split from atropine by an esterase, was converted to phenylacetaldehyde and phenylacetic acid.

It will be noted that the few cases where the breakdown products of alkaloids are precisely known include none strictly relevant to their catabolism in the plant, which remains a virgin field for biochemical study.

### VI. The functions of alkaloids in the plant

Many suggestions, based largely on teleological arguments, have been advanced to provide plausible, or at least possible, functions for alkaloids in the plants that produce them. Alkaloids have been variously considered as protection against attack by animals, insects, fungi, and parasitic angiosperms; as end-products of detoxification mechanisms, their deposition (in some species) in dead tissues being held analogous to excretion in animals; as nitrogenous reserves; and finally as more or less fortuitous by-products of nitrogen metabolism. None of these views is at all likely to be true in general, as might be expected from the varied chemical nature of alkaloids. Some may perhaps be true in particular cases. Alkaloidal plants found among the weeds that replace more palatable species in over-grazed pastures may owe their immunity to their alkaloids. Thorny non-alkaloidal plants are, however, equally prominent in such situations. The deposition of the very bitter berberine in the outer bark of several *Berberis* species has been considered a protection against animal attack (Chatterjee, 1943). Resistance to the root-rot fungus *Phymatotrichum omnivorum* in *Mahonia swaseyi* and *M. trifoliata* (Berberidaceae), and in *Sanguinaria canadensis* (Papaveraceae) is attributed (Greathouse & Watkins, 1938; Greathouse, 1939) to their alkaloids, which in culture inhibit the fungus in very low concentrations. Berberine is stated (Meisel & Pomoshchnikova, 1950) to be selectively absorbed in mitochondria of yeast, and to inhibit its respiration. Its effect on pathogenic fungi seems not to have been tested. Solanine is toxic to spores of *Fusarium caeruleum*, which causes dry rot in potato tubers, but seems unlikely to control the pathogen *in vivo* (McKee, 1959).

Protection against insects is not in general very effective; crops cultivated for the production of alkaloidal insecticides such as nicotine or anabasine are notoriously subject to insect attack, often by pests normally sensitive to their alkaloids. Resistant races of the pests seem

to develop readily. Much interest has been aroused by an apparent association between the resistance of *Solanum* species to larvae of the Colorado beetle (*Leptinotarsa decemlineata*) and their content of glycoalkaloids, especially demissine. It is still not clear how far variations in resistance are correlated with the amount and type of alkaloid present (Kuhn & Gauhe, 1947; Prokoshev & Petrochenko, 1950; Prokoshev, Petrochenko, & Baranova, 1952; Schreier, 1954, 1957).

Phanerogamic parasites flourish on at least some alkaloid-containing plants. Votchal (1889) noted the occurrence of *Cuscuta europaea* on *Solanum dulcamara*. The tissues of stems and leaves attacked by the parasite were found by microchemical tests to be unusually rich in solanine. This may represent a reaction to wounding; Molle (1895) found an increased solanine content in potato tubers after cutting. Votchal observed the fan-shaped absorbing ends of the *Cuscuta* haustoria to be almost as rich in solanine as the *Solanum* tissues in which they were embedded. Tissues of the *Cuscuta* stem at a short distance from the haustoria also gave colours with solanine reagents; the shades of colour were, however, atypical. Votchal suggested that either the solanine molecule was modified in the parasitic tissues, or other substances interfered with the colour reactions, but did not decide between the two possibilities. *Cuscuta* has been reported on other alkaloidal plants, e.g. *Atropa*, *Conium*, *Delphinium*, *Isotoma*, *Nicotiana* (Mirande, 1900; Gertz, 1915; Kindermann, 1928; Walzel, 1952a). Walzel (1952a), using highly sensitive microchemical methods, detected no nicotine in stems of *Cuscuta gronovii* parasitizing stems and leaves of *Nicotiana tabacum*; no special study was made of the haustoria of the parasite. *Cuscuta*, though a successful parasite of many alkaloidal plants, is severely affected by colchicine. Growing either on *Colchicum autumnale* or on colchicine-treated *Solidago canadensis* it produces abnormal haustoria from which tracheids are completely absent (Walzel, 1952b). Similar ineffective haustoria occur in *Cuscuta* growing on plants with latex or highly acid sap (Kindermann, 1928).

It appears that *Cuscuta* either does not absorb nicotine from tobacco plants on which it grows, or can destroy the alkaloid readily. Severe metabolic disturbances have been noted in *Atropa belladonna* seedlings absorbing nicotine from stocks of *Nicotiana glauca* (Hieke, 1942). Other Solanaceae not normally containing nicotine seem also to be injured by it when grafted to nicotine-producing stocks. Several broomrapes (*Orobanchaceae*, *O. cernua*, *O. indica*, *O. ludoviciana*, *Phelipaea ramosa*) attack field grown tobacco and may seriously reduce

its yield (Shaw, 1917; Izard, 1959). *O. muteli* growing on tobacco is stated (Zellner, 1919) to be free of nicotine.

Mistletoes (Loranthaceae) growing on *Duboisia myoporoides* absorb its alkaloids (hyoscyne, anabasine, isopelletierine) without apparent injury (Trautner, 1952; Mortimer, 1957). The latter author found in the mistletoe all the alkaloids detected in the host, but in lower concentrations on a fresh weight basis. *Dorophora sassafras*, which contains the alkaloid doryphorine (Petric, 1912), is a common host of the mistletoe *Korthalsella opuntia*. Another mistletoe, *Phrygilanthus eucalyptifolius*, is reported (Blakely, 1922) on alkaloidal Leguminosae (*Cytisus proliferus* and *Erythrina indica*).

Protection by alkaloids against the attacks of parasites or plant-eating animals, even if effective in some cases, can hardly be a general advantage to alkaloidal plants. Votchal (1889) rejected the general protection hypothesis for solanine on considering the numerous and successful insect enemies of the potato, but suggested that high solanine concentrations in young growing tissues gave protection where it was most needed. He produced no evidence, however, that these actively growing parts are in fact protected by the alkaloid. Attempts to establish such a function for other alkaloids also often involve unconvincing special pleading.

It has been suggested that alkaloids act as reserves of nitrogen. This is unlikely; their N/C ratio is low and they are mostly deposited in small amounts. Their metabolism seems to be parallel to that of proteins rather than a part of it, and it is on a very much smaller scale. In some germinating seeds there may be a transfer of nitrogen from alkaloid in the resting seed to protein in the seedling, but only a small part of the protein nitrogen could be supplied in this way. Alkaloids might more plausibly be considered as reserves of pre-formed heterocyclic rings required in the formation of co-enzymes and other essential substances. These rings, however, are formed effectively in non-alkaloidal plants. All autotrophic plants probably form the pyridine ring in nicotinic acid; comparatively few produce alkaloids containing it. Little is known about the effect of alkaloids on metabolic processes within the plant. Dawson (1946) reported increased absorption and reduction of nitrate in roots of tobacco plants grown in sand and supplied externally with nicotine; this was confirmed by Schmid (1948). The nature of the stimulus to nitrate metabolism is not known. It has been suggested that alkaloid formation removes from the cell free amino-acids that would otherwise be toxic, but there is no good

evidence for toxicity of the amino-acids concerned. Detoxification of ammonia, another suggested function for alkaloids, is supported at least by the known toxicity of ammonia. Alkaloids would, however, appear inefficient for its detoxification owing to the large amount of carbon required for their formation compared with the substances (asparagine, glutamine, citrulline, allantoin) normally storing surplus ammonia in a form more readily available for future use than in most alkaloids.

The only reasonable course, on the information at present available, is to consider the place of alkaloids in plant metabolism as largely unknown, and to renounce, on account of their great variability in structure and behaviour, any general explanation of their function. Their often spectacular effects in animals make it tempting to assume that they are equally potent in the plant. The temptation should be resisted. There are similarities between plant and animal metabolism, but also marked differences, and in animals alkaloids act largely on functions absent from plants. The pharmacological effects of alkaloids are probably responsible for the emphasis sometimes laid on their putative rôles in the plant; it should perhaps be remembered that equally little is known of the functions of other minor plant products, some of which, e.g. the terpenes, are equally complex in chemical structure.

## CHAPTER 13

# CYANIDES AND NITRO COMPOUNDS

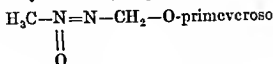
### (a) CYANIDE METABOLISM

Vauquelin (1800) reported apricot seeds to contain free hydrogen cyanide; it is, however, combined in a glucoside. Cyanogenetic glucosides, now known from several hundred species, are widespread in some families, e.g. Rosaceae, Gramineae, Compositae, Euphorbiaceae, and rare in others. Léeman (1935) listed 88 cyanogenetic grasses. Cyanogenetic species have been studied mainly because of their toxicity to stock or, rarely, to man; little is known about cyanide metabolism in the plant. Young plants, and particularly new shoots from established plants, are rich in cyanide, suggesting an association with active metabolism (Boyd, Aamodt, Bohstedt, & Truog, 1938; Winks, 1940; Franzke & Hume, 1945a). Leaves usually contain the highest concentration, but any plant part may be cyanogenetic, e.g. roots in cassava (manioc: *Manihot utilissima*, Euphorbiaceae) and flowers in *Grevillea banksii*, *Hakea saligna*, and *Lomatia silaifolia* (Proteaceae) (Smith & White, 1920), and *Lotus corniculatus* (Guérin, 1929). The cyanide content of plants is increased by high nitrogen supply (Boyd *et al.*, 1938) and by dry weather (Willaman & West, 1916). Ravenna & Peli (1907) found that sunshine increased cyanide in *Passiflora minima*, *Phaseolus lunatus*, and *Sorghum vulgare*. Detached sorghum leaves formed cyanide, apparently from nitrate, if illuminated or supplied with sugar in the dark. Healthy sorghum plants emit small amounts (0.26 mg/plant/day) of gaseous hydrogen cyanide (Franzke & Hume, 1945b), which is formed also in fruiting bodies of some higher fungi (Mirande, 1932; Heinemann, 1942). In *Pholiota aurea* (Bach, 1948) it arises by an enzymatic process requiring oxygen.

Most cyanogenetic glucosides yield on hydrolysis a ketone or an aromatic aldehyde as well as hydrogen cyanide. Amygdaloside (Wöhler & Liebig, 1837) from the almond yields benzaldehyde, and glucosides from *Phyllanthus gastroemii* (Euphorbiaceae) (Finnemore, Reichard, & Large, 1936) and *Zieria laevigata* (Rutaceae) (Finnemore & Cooper, 1936) contain respectively *p*- and *m*-hydroxybenzaldehyde. Cyanogenetic glucosides containing acetone occur in flax (*Linum usitatissimum*)

(Jorissen & Hairs, 1887) and many other species; a glucoside from *Lotus australis* contains methylethylketone (Finnemore & Cooper, 1938).

Some plant constituents of unusual structure yield hydrogen cyanide on relatively gentle chemical treatment. They include  $\beta$ -nitropropionic acid, known from several unrelated species, and macrozamin from leaves and seeds of eyeads (Cooper, 1940; Riggs, 1954). The latter is a primeverosyloxyazoxymethano (Langley, Lythgoe, & Riggs, 1951):



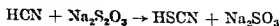
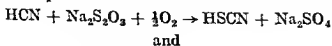
A few species, e.g. *Goodia lotifolia* (Leguminosae) (Finnemore & Large, 1936) and *Ribes fasciculatum* (Saxifragaceae) (Dillemann, 1954), liberate hydrogen cyanide from labile compounds of uncertain structure. The few known plant products that contain the nitrilo (CN) group but do not form glucosides include the growth substance indolyl-3-acetonitrile and the alkaloid ricinine (Fig. 63).

Formation of some cyanogenetic glycosides is associated with amino-acid metabolism. Gander (1958, 1959) showed in *Sorghum vulgare* that the nitrilo carbon of *p*-hydroxymandelonitrile- $\beta$ -glucoside arose from carbon atom 2 of tyrosine and suggested *p*-hydroxyphenylserine as an intermediate. Butler & Butler (1960) showed that in *Trifolium repens* valine was a precursor of linamarin and isoleucine of lotaustralin. Decarboxylation seemed to be involved, valine-4- $\text{C}^{14}$  but not valine-1- $\text{C}^{14}$  giving labelled linamarin.

In *Trifolium repens* (Williams, 1939; Corkill, 1942), and in interspecific crosses in *Linaria* (Dillemann, 1953), a single pair of genetic factors determines presence or absence of cyanide. Related species may vary greatly in cyanide content. *Heterodendrum oleaeifolium* (Sapindaceae) is highly cyanogenetic; the other species of the genus, *H. diversifolium*, is cyanide-free (Petric, 1920).

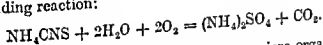
#### (b) THIOCYANATE METABOLISM

Lang (1933) found in animal tissues an enzyme (rhodanese) catalysing the formation of thiocyanate from thiosulphate and cyanide according to the equations





A similar reaction may occur in yeast (Bénard, Gajdos-Török, & Gajdos, 1947). Stoecklin & Crochetelle (1910) found thiocyanate in Cruciferae. Gemeinhardt (1938) detected it in all of 54 plants, the richest being crucifers and umbellifers; he suggested it was formed by the rhodanese reaction, both cyanide and thiosulphate being known in plants. Wood & Fiedler (1953) stated  $\beta$ -thiolpyruvate to be a substrate for rhodanese; its reaction with cyanide to form thiocyanate is now attributed to a distinct enzyme,  $\beta$ -thiolpyruvate transulphurase (Sörbo, 1954; Kun & Fanshier, 1959). This enzyme, known as yet only from animal tissues, is a copper protein and transfers sulphur from  $\beta$ -thiolpyruvate to sulphite, forming thiosulphate, and to cyanide, forming thiocyanate. The further metabolism of thiocyanate in plants is obscure. Ammonium thiocyanate serves as the sole source of carbon for *Bacillus thiocyanoxidans*, isolated from gas-works effluents by Happold & Key (1937). The thiocyanate is oxidized to sulphate by the energy-yielding reaction:



Waro & Painter (1955) isolated from sewage a micro-organism using, as its sole source of carbon and nitrogen, cyanide which was apparently converted quantitatively to ammonia. Hydrogen cyanide, if supplied together with sucrose, is a good nitrogen source for the mould *Aspergillus niger* (Ivanov & Osnitskaya, 1934). Several workers (Dzeani, 1913; Sanford, 1914; Elliot, 1917) inserted solid or dissolved cyanides into plant stems to kill insect pests. The cyanide was apparently rapidly metabolized to undetermined products.

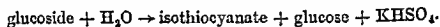
In higher plants cyanogenetic glucosides seem to be metabolized, but little is known of the processes involved or of their physiological significance. Godwin & Bishop (1927) reported a marked reduction in the cyanogenetic glucoside content of starving detached leaves of cherry laurel (*Prunus laurocerasus*). A similar decrease was observed during drying in leaves of *Indigofera galeoides* (Treub, 1909). Cyanide disappeared in macerated tissues of *Prunus* spp. (Alsberg & Black, 1916), *Tridens flavus* (Viehoever, Johns, & Alsberg, 1916), *Arum maculatum*, and *Linaria striata* (Dilleman, 1953); hydrogen cyanide did not seem to be lost by volatilization.

Turrell & Weber (1955), using  $\text{S}^{35}$  as a tracer, showed that elemental sulphur dusted on to lemon leaves was absorbed and assimilated into protein. A probably enzymatic reduction of elemental sulphur to hydrogen sulphide is reported in extracts from yeast and higher plants

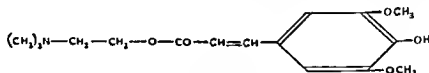
(de Rey-Pailhade, 1888*a, b*, 1897; Deleano, 1909); Pozzi-Escot (1902) recorded reduction in this way of both selenium and sulphur. The metabolism of elemental sulphur in higher plants is obscure; rhodanese or a similar enzyme may catalyse its reaction with cyanide to form thiocyanate.

### (c) ISOTHIOCYANATES IN PLANTS

These compounds cause the characteristic flavour of "mustard oils" in various Cruciferae; they occur also in similarly tasting products from quite unrelated families, e.g. seeds of *Carica papaya* (pawpaw) and leaves of *Tropaeolum* (garden nasturtium). In the plant they occur as glucosides. The first of these to be isolated were sinalbin (Boutron & Robiquet, 1831) from *Sinapis alba* (white mustard) and sinigrin (Bussy, 1840) from *Brassica nigra* (black mustard). The glucosides are accompanied in the plant by an enzyme (myrosinase) hydrolysing them according to the following equation:



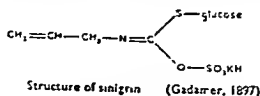
Sinigrin yields allyl isothiocyanate (Will, 1844) and sinalbin the *p*-hydroxybenzyl compound (Salkowski, 1889). In sinalbin potassium sulphate is replaced by the sulphate of an organic base, sinapine (Fig. 87).



Sinapine

FIG. 87.

The structure (Fig. 88) put forward by Gadamer (1897) was long accepted for these glucosides, but has now been replaced by that of Ettlinger & Lundeen (1956*b*) (Fig. 89). Strong support for this formula is given by the first synthesis of a mustard oil glucoside (Ettlinger &



Structure of sinigrin (Gadamer, 1897)

FIG. 88.



natural sources. It is now known from *Viola odorata* (Pailer & Nowotny, 1958) and from *Indigofera endecaphylla* (Morris, Pagán, & Warmke, 1954); it may not, however, be the main toxic constituent of the latter species (Hutton, Windrum, & Kratzing, 1958). It is also a metabolite of the moulds *Aspergillus flavus* (Bush, Touster, & Brockman, 1951) and *Penicillium atroventum* (Raistrick & Stössl, 1958). In *P. atroventum* over 60 per cent of the nitrogen of ammonia metabolized by the actively growing mould, apart from that incorporated into the mycelium, was recovered from the medium as  $\beta$ -nitropropionic acid; its production was ten times as great with ammonia as with nitrate, suggesting an active oxidation of reduced nitrogen compounds. *Aristolochia clematitis* contains the more complex nitro compounds 3,4-methylenedioxy-10-nitrophenanthrenecarboxylic acid and its 8-methoxy derivative (Pailer, Belohlav, & Simonitsch, 1955, 1956; Pailer & Schleppnik, 1957). The former occurs also in *A. reticulata* and *A. indica* (Coutts, Stenlake, & Williams, 1957) and in *A. bracteata* (Rao, Row, & Murty, 1959). The fungus *Clitocybe suaveolens* forms a nitroso derivative of benzaldehyde (Herrmann, 1960). *Streptomyces lavendulae* produces the well-known antibiotic chloramphenicol (chloromycetin), a derivative of nitro-phenylserine (Rohstock, Crooks, Controulis, & Bartz, 1949).

## CHAPTER 14

# STORAGE AND TRANSPORT OF NITROGENOUS SUBSTANCES

### A. Nitrogenous compounds in vegetative storage organs

In trees and other woody plants the living parenchymatous tissues of the stem contain reserve materials and are the only storage organs. Perennial herbaceous plants have more varied and more specialized storage organs, arising by modification of various parts of the plant body. The familiar bulbs of onion (*Allium cepa*) or various species of tulip (*Tulipa*) or lily (*Lilium*) are characteristic of some monocotyledonous families; they occur in some dicotyledons, e.g. *Oxalis latifolia* and *O. martiana*, but are rare in this group. In the bulb the storage tissue consists of modified leaf bases surrounding an apical bud borne on a greatly reduced and flattened stem. Other underground storage organs are modified roots or rhizomes (horizontal stems often growing underground). The aerial pseudobulbs found in many orchids are short swollen stems or branches borne at the base of the leaves.

Some trees, e.g. the baobabs (*Adansonia*, Bombacaceae) and the bottle tree (*Brachychiton rupestris*, Sterculiaceae), store large amounts of water in swollen stems. Others store great quantities of starch, especially monocarpic species, which use materials accumulated over many years to produce a huge inflorescence, the tree dying after fruiting. This habit is found in some palms, including *Metroxylon sagu* and *M. rumphii*, whose trunks yield the sago of commerce. Monocarpy is rare in dicotyledonous trees, but probably occurs in *Cerberiopsis candelabrum* (Apocynaceae), a species common in New Caledonia. The pith of *Metroxylon sagu* (raw sago) has a very low nitrogen content: samples from New Guinea contained 0.035 per cent nitrogen on a fresh weight basis (0.05 per cent dry weight) (Peters, 1959). Other species of palm may, however, store substantial amounts of nitrogen in the stem. Gallerand (1904) found "albuminous matter" to represent 10.5 per cent of the dry weight in a sago-like pith from the satranabe palm of Madagascar (*Medemia nobilis*). Total nitrogen in this pith must be about 1.7 per cent of the dry weight, over thirty times as much as in sago. The sap flowing from cut inflorescence stalks of annually flowering

palms, particularly *Borassus flabellifer*, *Cocos nucifera*, and *Nipa fruticans*, contains much soluble carbohydrate and is a major source of sugar in some parts of Asia. The sap from cut inflorescence stalks of the coconut palm (*Cocos nucifera*) contains 0.05 per cent of nitrogen; the daily loss of nitrogen per tree is 0.5 to 2.4 g (Browning & Symons, 1916).

Vegetative storage organs contain the same type of nitrogenous compounds as other parts of the plant, but have characteristically a high proportion of soluble nitrogen and a correspondingly low proportion of protein. Schulze & Urieh (1875) showed that in roots of turnip (*Brassica napus* var. *napobrassica*) protein represented about 20 to 40 per cent of the total nitrogen; much of the soluble nitrogen was present as amino groups. Subsequent work (Schulze & Barbieri, 1880; Schulze & Eugster, 1882; Schulze, 1904b) demonstrated the presence in potato tubers of several individual amino-acids, including arginine, histidine, leucine, lysine, and tyrosine. Glutamine was found, sometimes in comparatively high concentrations, in roots and tubers of beet (*Beta vulgaris*), carrot (*Daucus carota*), radish (*Raphanus sativus*), celery (*Apium graveolens*), *Stachys tubifera*, kohlrabi (*Brassica oleracea* var. *gongylodes*), and turnip (Schulze & Bosshard, 1888; Von Planta, 1890; Schulze, 1896b, 1898). Gruntuch (1929) reported high contents of soluble nitrogenous substances in underground storage organs of numerous plants, including species of *Allium*, *Asparagus*, *Canna*, *Dahlia*, *Helianthus*, and *Oxalis*. Kinoshita (1897c) found asparagine to represent 2 per cent of the dry weight in roots of *Nelumbo nucifera* (Nymphaeaceae). Ishizuka (1897) showed that the asparagine content increased in roots of *Brassica campestris*, *Daucus carota*, and *Raphanus sativus* examined after storage for 60 and 100 days at ambient temperature. More recent work (Dent, Stepka, & Steward, 1947; Steward, Thompson, & Dent, 1949; Payne, Fuhs, & Hay, 1952; Thompson & Steward, 1952; Zacharius, Thompson, & Steward, 1952) using paper chromatography has shown that the soluble nitrogen of potato tubers contains most of the amino-acids commonly found in protein, together with others ( $\gamma$ -aminobutyric acid,  $\beta$ -alanine, pipercolic acid) which are absent from most and perhaps from all proteins whose composition is completely known.

Glutamine, asparagine, and arginine are quantitatively the most important constituents of the soluble nitrogen in potato (Thompson & Steward, 1952). These three substances are also prominent in cassava tubers (*Manihot utilissima*) (Van Veen & Lanzing, 1941; Bigwood, Adams, & McDard, 1952). The protein content of cassava tubers is

ally less than 1 per cent of the dry weight (Jacquot & Nataf, 1936; Ramamurthy, 1945; Peters, 1959). Much higher protein contents (per cent to 7 per cent) are recorded (Ammann, 1920) for certain species grown in Cambodia, but seem to be very unusual in cassava. In sprouting potato tubers much of the soluble nitrogen is transferred to the developing shoots (Street, Kenyon, & Watson, 1946c); glutamine content of the tuber falls greatly at this stage. Protein is affected, suggesting that it is not readily mobilized for use in living tissues.

Reuter (1957a) made an extensive chromatographic study of the soluble nitrogenous constituents of vegetative storage organs in 166 species. The main compounds in the majority of these species were glutamic acid, aspartic acid, and their amides. These were, indeed, found in almost all species, but in some they were only minor constituents associated with larger amounts of other compounds.  $\delta$ -N-tylosin was the main soluble nitrogenous reserve compound in 19 species of Fumariaceae examined; it is also known from another species of this family (Manske, 1937). Reuter (1957a) found it only in Fumariaceae and in 4 species of the related family Papaveraceae. The distribution thus seemed to have a restricted and well-defined taxonomic basis. Arginine predominated in many species; they tended to be concentrated in the family Rosaceae but some belonged to other families. Species accumulating proline were numerous in Leguminosae, e.g. *Amorpha paniculata*, *Robinia pseudacacia*, *Sophora japonica*; they were scattered through other families. Proline is also a major component of the soluble nitrogen in species of *Citrus* (Rutaceae) (Giri, Gopalakrishnan, Radhakrishnan, & Vaidyanathan, 1952; Raveux, Bové, & Bové, 1957) and of *Santalum* (Santalaceae) (Giri *et al.*, 1952; Kee & Urbach, 1955); it is the main amino-acid in dormant buds of *Juniperus communis* (Rosaceae) (Cronenberger, 1959). Citrulline predominated in species of Betulaceae and Juglandaceae; it was prominent also in *Veronica refracta* (Iridaceae), *Calycanthus occidentalis* (Calycanthaceae) and *Brassica oleracea* (Cruciferae). Bollard (1957c) recorded citrulline as a major constituent of a few unrelated species. Azetidine-2-carboxylic acid forms about 75 per cent of the non-protein nitrogen in the roots and rhizomes of *Convallaria majalis* and *Polygonatum multiflorum* (Liliaceae) (Fowden & Bryant, 1938; Fowden, 1959a); it is also conspicuous in storage organs of *Borica volubilis* (Fowden & Steward, 1957a; Reuter, 1957a). Other amino-acids reported as major constituents in

some species include alanine,  $\gamma$  aminobutyric acid, leucine, phenylalanine, serine, and valine. There is thus considerable variety in the compounds storing nitrogen in different species. Some, like asparagine and glutamine, are very widespread, others, like azetidine 2 carboxylic acid, are known only from a group of related species, but may yet be found in unrelated plants.

## B Translocation of nitrogenous compounds

### (a) PATHWAYS OF TRANSLOCATION

It has long been clear that in higher plants soluble substances move rapidly both upwards from the roots and downwards from the leaves. Increasing recognition of the synthetic activities of the root system has further emphasized the mobility of materials within the plant. Simultaneous movement in both directions complicates experimental study. Phillips & Mason (1936a) and Fischer (1936) showed that over fairly long periods (sampling at intervals of two days or more in an experiment lasting two weeks) carbohydrates moved downwards in plants while nitrogen moved upwards. Their conclusion that these substances were simultaneously transported in opposite directions in the phloem was, however, rendered uncertain by the long duration of the experiments. More definite evidence of simultaneous transport upwards and downwards in the plant was given by Chen (1951), who showed that in geranium plants (*Pelargonium*) and willow cuttings (*Salix*) inorganic phosphate labelled with  $P^{32}$  was transported upwards from the roots through the phloem of the stem, at the same time radioactive sugars formed in the leaves from  $C^{14}$  labelled carbon dioxide were moved downwards, also in the phloem. There are, however, periods in the life history of both annual and perennial plants when transport operates predominantly in a single direction. Well known examples include the flow of soluble materials from aging leaves and to developing seeds.

Kursanov and his associates found an active and rapid circulation of materials between different organs of seedlings. Carbohydrates pass from the leaves to the roots where they are metabolized to compounds, presumably keto acids which provide the carbon skeletons of amino acids. The amino acids synthesized in the roots are in part exported to the shoot. Detached shoots of wheat take up amino acids efficiently from solution through the cut end of the stem (Kursanov & Zaprometov, 1943a, b, Kursanov, Kryukova, & Sedenko, 1948, Kursanov, 1952), if



ripening ears are present they receive most of the absorbed amino-acids. This transport of amino-acids requires respiratory energy; this may explain the high respiration rates of vascular tissues (Kursanov & Turkina, 1952a, b; Willenhrink, 1957).

Active synthesis of numerous amino-acids in roots has been demonstrated in a wide range of species (Willis, 1951; Kursanov, Tuyeva, & Vereshchagin, 1954; Kursanov, 1955; Mothes & Engelbrecht, 1956; Yemm & Willis, 1956; Kulayeva, Silina, & Kursanov, 1957). The accumulation of amino-acids in actively growing aerial roots of figs (*Ficus*) is particularly striking (Kursanov, 1955). The roots, though important in amino-acid synthesis, are not the only seat of this process. There is abundant evidence (e.g. Bidwell, Krotkov, & Reed, 1954; Voskresenskaya, 1956) that amino-acids are formed in leaves, and that they can be exported from them to other parts of the plant (Carles, 1958).

#### (b) TRANSLOCATION AWAY FROM LEAVES

Several early workers (e.g. Borodin, 1876; Pfeffer, 1876; Schulze, 1880) conjectured that formation and breakdown of proteins both occur continuously in the leaf. These processes were envisaged as being primarily related to respiration, but protein hydrolysis in normal attached leaves could also provide soluble nitrogenous compounds, particularly amino-acids, for transfer to other parts of the plant. Suzuki (1898a) concluded from analyses at different times of the day on leaves of *Eragrostis esculentum*, *Helianthus annuus*, *Ipomoea batatas*, *Phaseolus mungo*, *P. vulgaris*, *Pueraria thunbergiana*, *Solanum tuberosum*, and *Wistaria brachybotrys* that during the day protein was synthesized from nitrate, while at night hydrolysis predominated, amino-acids and asparagine being translocated away from the leaves.

Some early reports on this subject are contradictory and difficult to interpret as the results are often expressed on a dry weight basis; changes in nitrogen content were thus liable to be obscured by concurrent changes in carbohydrates. The choice of a suitable basis for the expression of results is both important and difficult in such work. The absolute amount of nitrogen or protein per leaf is perhaps the best basis of comparison, though variability between leaves makes large samples desirable. Schulze & Schutz (1909) showed on this basis that leaves of *Acer negundo* had more total and protein nitrogen in the evening than in the early morning. This effect was consistently shown by young and mature leaves at five sampling dates; senescent leaves,

however had less total and protein nitrogen in the evening, protein hydrolysis and translocation appearing to predominate even during the day Chinnall (1924a, b) found that protein content in leaves of the runner bean (*Phaseolus multiflorus*) decreased at night, and deduced from his observations a diurnal variation in relative rates of protein synthesis and hydrolysis, the latter predominating at night Maskell & Mason (1929) obtained similar results with the cotton plant (*Gossypium*) Smirnov, Erygin, Drboglav, & Mashkovtsev (1929) presented very extensive data on changes of total and protein nitrogen in leaves of tobacco (*Nicotiana tabacum*) and sunflower (*Helianthus annuus*) Their results were expressed as mg N per square m of leaf surface In mature leaves, protein nitrogen per unit area even increased during the day, the increase with young leaves was smaller, but would have appeared greater on an absolute basis, as these leaves were still growing The nitrate content was much higher in young than in mature leaves suggesting that the former, although further from the source of nitrate in the soil, absorbed it more effectively In both young and old leaves protein content fell in the middle of the day rising steeply in the afternoon to pass the level reached in the early morning Smirnov *et al* (1929) attributed this decrease to high mid-day temperatures, Mothes (1926) showed a fall of protein content in leaves of plants exposed to high temperatures, presumably because hydrolysis was accelerated more than synthesis Studies on the effects of various nutrient deficiencies on the nitrogenous metabolism of barley (*Hordeum*) leaves (Richards & Templeman, 1936, Gregory & Sen, 1937) gave further evidence that leaf protein was not metabolically inert, but could readily be mobilized by hydrolysis The conclusion that some proteins at least are active metabolites has since been confirmed in experiments with isotopic nitrogen (e.g. Hevesy, Linderström Lang, Keston, & Olsen 1940, Turchin, Gumin, Laya & Plyshevskaya 1953) The latter authors showed that the nitrogen of chlorophyll is also continually renewed The evidence for continuous turnover of proteins in some tissues seems clear, but it is not yet certain how far this applies to proteins in general

The age of a leaf markedly affects its protein metabolism The protein content of young leaves increases as they grow but in older leaves protein is hydrolysed and its breakdown products are translocated to other parts of the plant In different parts of a mature plant there are tissues at all stages of development senescent organs releasing materials used in new growth In most annual plants some leaves are

shed comparatively early in development, long before flowering. The withering of leaves on senescent annual plants and leaf-fall in deciduous trees at the beginning of their dormant season (winter in temperate climates, hot dry summers in the arid tropics) are striking examples of shedding short-lived organs. Leaves of evergreen trees are also temporary structures, though their life-cycle is less obvious than in deciduous species, and has been less studied. Some evergreen trees shed and replace leaves steadily all the year round, others show periods of comparatively rapid leaf-fall followed by flushes of new growth, perhaps several times a year.

The return of nitrogen from the leaves of deciduous woody plants to permanent storage organs (usually the stem) has been studied by many workers. Sachs (1865) concluded from the decrease of starch and chlorophyll in senescent leaves that materials must be returned to the perennial part of the plant. Leclerc Du Sablon (1904, 1906) showed that nitrogenous materials were transferred in spring from stems and roots to the developing buds and young leaves; he also found a return of nitrogen to the perennial organs from senescent leaves in autumn. Richter (1910) found with apple, cherry, pear, and plum trees that the nitrogen content per leaf remained fairly steady through the late summer and early autumn months (July to early October). In the later part of October it fell rapidly. The nitrogen remaining at leaf-fall varied among these species from 23 per cent to 32 per cent of the maximum value recorded. Other work at this period was summarized by Combes (1911); much of it was difficult to interpret because the data were expressed solely on a dry weight basis; the earlier work is also thoroughly discussed by Combes (1926) and Échevin (1931).

Combes (1924) showed that loss of nitrogen from yellowing leaves was not, as had been suggested, due to leaching of soluble compounds by rain; detached leaves exposed to the weather retained much more nitrogen than controls attached to the plant. Nitrogen may not be leached from leaves to any significant extent. Appreciable losses of potassium from leaves washed by dew have, however, been recorded (Arens, 1934; Phillis & Mason, 1942a). Later work by Combes and his associates clarified the movement of nitrogen by analysis throughout the year of entire woody plants; two-year old oaks (*Quercus*) and beeches (*Fagus sylvatica*) were mostly used (Combes, 1926, 1927; Combes & Échevin, 1927; Combes & Piney, 1928, 1929). Protein hydrolysis in stems and roots began in February, two months before the leaf buds opened, and continued until May, when a period of net

protein synthesis in these organs began. This accumulation of protein continued until the time of leaf fall in November, when for a brief period hydrolysis predominated in roots and stems as well as in leaves. At this stage the total nitrogen content of the plant decreased, probably by excretion of nitrogenous substances through the roots. The nature of this loss of nitrogen is obscure, it has been observed in other plants, especially annuals (Wilfarth, Römer, & Wimmer, 1906, Burd, 1919, Penston, 1935, Deleano & Gotterbarm, 1936, Mothes & Engelbrecht, 1952a).

In some cases at least this decrease in total nitrogen cannot be attributed to leaf fall or loss of other plant parts, or to transfer of nitrogenous substances towards the roots. Knowles & Watkin (1931) found that wheat plants attained their maximum nitrogen content three weeks before harvest, no change in total nitrogen occurred thereafter, though transfer to the ear continued. Over the last three weeks before harvest the above ground parts of the plant lost substantial amounts of all elements studied, except nitrogen and phosphorus, losses of calcium, potassium, and chlorine were particularly marked. Leaf fall and leaching were eliminated as causes for these losses, they may have been due in part to transfer to the roots, which were not analysed. Luttkus & Böttcher (1939) showed that darkening induced a substantial excretion of inorganic materials through the roots of maize plants grown in culture solution. Up to 30 per cent of the total potassium of the plant was lost in this way, sulphate and phosphate were also excreted. No damage to the roots was observed.

Gaumann (1935) recorded extensive analytical data on the distribution of nitrogen in different parts of young beech trees throughout the year. The total nitrogen content of the leaves increased very rapidly during May. It remained roughly constant from the end of May to the middle of October, and then fell steeply. The rate of loss of nitrogen in autumn was however always less than the rate of intake in spring. In leaf buds and young leaves soluble nitrogenous compounds were rapidly condensed to protein up to the end of May, when synthesis slowed down and there was a period of net hydrolysis, followed by net synthesis again until July. Yellowing leaves lost 50 per cent or more of their nitrogen in the three weeks preceding leaf fall. Similar observations are recorded for *Salix fragilis* (Deleano & Andreesco, 1932, Mcrop, 1936) and for *Vitis vinifera* (Alexander, 1957). Numerous authors have recorded increased protein content in stems particularly in the bark of woody plants in the autumn, e.g. Murneek & Logan

(1932) for apple (*Pyrus malus*) and Siminovitch & Briggs (1949) for *Robinia pseudacacia*. Leaves of evergreen plants have been less studied, but Michel-Durand (1932) found that the same proportion (40 per cent) of their maximum nitrogen content remained in yellow fallen leaves of *Prunus laurocerasus* (evergreen) and *Costanea vulgaris* (deciduous). Both species also lost the same proportion of potassium (60 per cent) in fallen leaves. The relative amounts of sulphur and phosphorus lost in fallen leaves were, however, much higher in the evergreen species. Hannon (1956) recorded that sclerophyllous leaves of *Angophora costata* and cladodes of *Casuarina littoralis* lost no nitrogen before falling from the tree.

In annual plants mature and to a greater extent senescent leaves tend to hydrolyse protein and export its soluble products to metabolically more active parts of the plant. Mature leaves of barley (*Hordeum*) (Walkley, 1940; Walkley & Petrie, 1941) and of cotton (*Gossypium*) (Phillis & Mason, 1942b) are, however, still capable of protein synthesis. Walkley (1940) used the fourth leaf of the main shoot on barley plants, the upper part of the main shoot and all tillers being removed; a high supply of nitrogen as ammonium sulphate was provided via the roots. In these conditions protein synthesis was rapid even in senescent leaves, provided they still retained some chlorophyll. Similar results are reported for other species, e.g. tobacco (Mothes, Böttger, & Wollgeln, 1958). Even in detached leaves that usually show rapid loss of protein, some synthesis continues and, though masked by concurrent hydrolysis, can be detected with isotopic nitrogen (Chibnall & Wiltshire, 1954). Detached senescent leaves are metabolically rejuvenated by the formation of adventitious roots. Rooted senescent leaves of *Nicotiana* and *Phaseolus* show renewed plastid formation, synthesizing protein, nucleic acids, and chlorophyll and accumulating materials absorbed or synthesized by the roots (nitrate, glutamine, allantoin, nicotine) (Mothes & Engelbrecht, 1956; Mothes, Böttger, & Wollgeln, 1958).

### (c) TRANSLOCATION IN DEVELOPING FLOWERS

Schumacher (1931-32) demonstrated a remarkably rapid breakdown of protein in the perianth of ephemeral flowers of various species. These flowers, though often large and showy, are very impermanent structures, withering a few hours after they open. The maximum protein content is often in the bud just before opening; hydrolysis begins as the flower opens and may break down a considerable part of the protein before any sign of withering appears. To quote Schumacher: "Protein

synthesis stops as the flower opens; the machine is switched off, and while we admire the wonderful beauty of the unfolding flower, the secret deadly process of protein breakdown proceeds in its vitals, and after reaching a certain point can end only in catastrophic collapse." In ephemeral flowers of *Hydrocleis nymphoides* (Butomaceae), 28 per cent of the original protein broke down in 15 minutes, and a further 14 per cent in the next 45 minutes. This sudden breakdown has its counterpart in the rapid increase of protein and total nitrogen in developing flower buds, as has been emphasized by Combes (1935), who analysed the various floral parts of *Lilium croceum* at different stages of development. In cotton (*Gossypium*), which has short-lived flowers, there is a considerable import of nitrogen, together with phosphorus, potassium, magnesium, and chlorine, into the corolla during the night before anthesis; a corresponding export to the stem via the peduncle occurs on the following night. Transport in each direction appears to take place in the phloem (Phillips & Masou, 1936b). The total nitrogen content of inflorescences of *Acer pseudoplatanus* growing from the bud to the flowering stage increases about six times (Brunel & Échevin, 1938). In this species the glyoxylic ureides allantoin and allantoic acid account for a large part of the soluble nitrogen, and are much more prominent than the amides. The intense metabolic activity of the flower at anthesis is also shown, in *Iris germanica* and *I. flavescens*, by a sharp peak in respiratory activity at this time (Ulrich & Paulin, 1957).

The protein content of unpollinated orchid flowers remains steady for up to seven days, but pollination is followed by rapid changes (Schumacher, 1931-32; Gessner, 1948; Hsiang, 1951). The nitrogen content of the flower as a whole does not necessarily fall, but it is redistributed among the floral parts, passing from the labellum and the sepals to the ovary and gynostemium (column). The stimulation of metabolic activity is also shown (Britikov, 1951) by a great increase in the rate of uptake of  $P^{32}$ -labelled phosphate by the pistil of maize after pollination. In many species with ephemeral flowers more than half of the nitrogen liberated by protein breakdown in the petals passes to other parts of the plant before they fall, as found in *Althaea rosea*, *Cereus macdonaldiae*, *Convolvulus sepium*, *Datura metel*, *Pharbitis hispida*, and *Tigridia pavonia* (Schumacher, 1931-32). In *Lilium croceum* the pistil gained nitrogen steadily, while rapid protein hydrolysis took place in the perianth, the nitrogen gained by the pistil was, however, only 9 per cent of that lost from the perianth (Combes, 1935).

In detached inflorescences of *Iris* there is a striking transfer of material between different flowers. Ulrich & Paulin (1957) found the opening of the flower to be accompanied by a marked uptake of water and of mineral substances. In detached inflorescences of three flowers picked in bud and supplied with water through the stalk, all the flowers opened, the terminal bud opening first. If the inflorescence was held without water, the terminal flower failed to open, but the lowest bud did open, drawing water and other substances from the stem and from the terminal flower. The experimental conditions thus reverse the normal flow of materials.

#### (d) THE FLOW OF MATERIALS TO DEVELOPING FRUITS AND SEEDS

It has long been recognized, from quantitative analyses by early workers, that developing fruits and seeds draw on other parts of the plant for the supplies of nitrogen used in their growth. This flow of materials towards the seeds is particularly marked in annual plants. It may be noted that most workers on the physiology of seed development have studied crop plants selected for high seed production and belonging to large-seeded species. The available information on the redistribution of nitrogen in seed formation is based largely on work with members of the Leguminosae (pulses) and Gramineae (cereals), which are convenient for experiment and have seeds of economic importance. There are, however, some data for tobacco (Solanaceae), a plant not cultivated primarily for its seeds, and for trees.

The total nitrogen content per plant increases over at least the early part of fruit growth in annual plants. Boussingault (1846) estimated the nitrogen content (in kg/ha) of a crop of wheat as 12.4 on 19 May, 23.7 on 9 June (flowering,) and 42.0 on 15 August (harvest). Analysis of various organs of the plant at successive stages of growth indicates, however, that although some of the nitrogen used in the growing fruit comes directly from the roots, much is transferred from the stem and from senescent leaves. The flow of nitrogen from stems and leaves to the fruit appears in the data of Arendt (1859) for oat plants analysed at various stages of development. Anderson (1866b) sampled a crop of beans (*Vicia faba*) near Glasgow at various dates during 1864, and analysed separately roots, stems, leaves, flowers, and fruits. His analyses were very extensive, including water, total solids, iron, calcium, magnesium, sodium, potassium, sulphur, phosphorus, silica, and nitrogen; only the last need concern us here. The results are expressed in lb/acre. The experimental plot is stated to have contained 100,125

plants per acre; it is thus possible, assuming that this number remained constant over the growing season, to convert the results to the more convenient form of mg/plant (Table 11). The nitrogen contained in the

TABLE 11

*Changes in total nitrogen (mg/plant) in various parts  
of the bean plant (Vicia faba) during growth  
(Calculated from data of Anderson, 1866b.)*

	<i>Date of sampling (1864)</i>					
	1 June	1 July	1 Aug.	1 Sept.	7 Oct.	8 Nov.
<i>Roots</i>	7	56	54	73	78	74
<i>Stems</i>	7	77	298	333	195	178
<i>Leaves</i>	21	117	346	338	158	
<i>Flowers</i>		15	28			
<i>Fruits</i>			21	226	405	416
<b>TOTAL</b>	35	265	747	970	820	668

roots showed no significant decrease up to the last analysis, which was made in November because the crop matured late, owing to the apparently particularly poor summer. Between the beginning of August and the beginning of September the nitrogen content of the fruits increased markedly without any significant reduction in that of the stems and leaves. Nitrogen in the whole plant increased over this period, any translocation to the young fruits from stems and leaves being replaced from the soil via the roots or from the atmosphere via the root nodules. Later, between the beginning of September and the beginning of October, nitrogen lost from the stems roughly equalled that gained by the fruits. There was also a substantial loss of nitrogen from the leaves over this period, but it may largely have been due to leaf-fall; the leaves at the last sampling, early in November, were described as "a few blackened and moist fragments". The percentage of the nitrogen of the whole plant contained in different organs is shown in Table 12; the steep rise in the proportion of nitrogen laid down in the fruit is very striking. Fruhling & Grouven (1867) deduced from analyses of plants at various stages of growth that developing fruits and seeds use nitrogenous materials stored previously in other organs and as other chemical compounds. They studied 12 species, mostly cereals and leguminous fodder plants; results are given only as percentages, which reduces their quantitative value.

Emmerling (1880, 1887, 1900) grew *Vicia faba* at Kiel in the years



1879 and 1880. Samples of roots, stems, leaves, and, in the later stages, hulls and seeds were taken throughout the growing season. Analysis of the dried samples and study of a vast mass of data occupied Emmerling for the next twenty years. He recorded for each part at each sampling date the content of many different nitrogen fractions, not all of which are easily interpreted in terms of present-day concepts. The analyses were highly laborious, depending almost entirely on gravimetric or gasometric methods. The data were expressed both on a fresh-weight or dry-weight basis, and as amounts of the various constituents per thousand plants. The amounts per seed and per hull in growing fruits were not stated directly, but for most samples data were given from

TABLE 12

*Percentage of the total nitrogen of the bean plant (Vicia faba) contained in various parts during growth.*  
(Calculated from data of Anderson, 1866b.)

*Date of sampling (1864)*

	1 June	1 July	1 Aug.	1 Sept.	7 Oct.	8 Nov.
<i>Roots</i>	20	22	7	8	9	11
<i>Stem</i>	20	30	40	34	24	27
<i>Leaves</i>	60	42	46	35	19	
<i>Flowers</i>		6	4			
<i>Fruits</i>			3	23	48	62

which they could be calculated. The expression of the results on this basis often provides a clearer picture of changes in developing organs, in particular of the relations between protein and non-protein nitrogen, than is possible on a dry-weight or fresh-weight basis alone. Data expressed only per unit dry-weight or fresh-weight may mask relationships apparent on a per plant or per organ basis, which eliminates the effect of other processes going on concurrently, e.g. large accumulations of non-nitrogenous solids in developing seeds or loss of water in the later stages. Many workers (e.g. Arendt, 1859; Pfeiffer, 1876; Delcane & Bordeianu, 1933; Vickery, Pucher, Leavenworth, & Wakeman, 1935) have stressed this point, but it remains worthy of mention as even now some papers report developmental changes in composition on a dry-weight or fresh-weight basis only.

Some aspects of the work by Emmerling are summarized in Table 13 (absolute amounts) and Table 14 (distribution of nitrogen between different organs). In the early stages of growth about 60 per cent of the

## STORAGE AND TRANSPORT

TABLE 13

*Changes in total nitrogen (mg/plant) in various parts of the bean plant (Vicia faba) during growth.*  
(Tabulated from data of Emmerling, 1900.)

*Date of sampling (1880)*

	25 May	9 June	12 July	26 July	10 Aug.	30 Aug.	10 Sept.	23 Sept.
<i>Roots</i>	9	14	21	26	32	39	43	
<i>Stems</i>	5	18	45	56	68	85	94	
<i>Leaves</i>	21	45	149	158	161	102	57	
<i>Hulls</i>			14	54	63	37	36	
<i>Seeds</i>			4	60	156	436	442	446
<b>TOTAL</b>	35	77	233	352	520	699	672	—

nitrogen of the plant was in the leaves; this proportion fell rapidly once fruit development started and nitrogen was laid down in the seeds. Some nitrogen may also have been lost in fallen leaves. The absolute nitrogen content of roots and stem increased steadily throughout the experiment; their proportion of the total nitrogen of the plant declined owing to more rapid increase in the fruits. In the early stages of fruit development, nitrogen accumulated in the hulls; later it decreased

TABLE 14

*Percentage of the total nitrogen of the bean plant (Vicia faba) contained in various parts during growth.*  
(Calculated from data of Emmerling, 1900.)

*Date of sampling (1880)*

	25 May	9 June	12 July	26 July	10 Aug.	30 Aug.	10 Sept.
<i>Roots</i>	25	18	9	7	6	6	6
<i>Stems</i>	15	23	19	16	13	12	14
<i>Leaves</i>	60	59	64	45	31	15	8
<i>Hulls</i>			6	15	12	5	5
<i>Seeds</i>			2	17	33	62	67

there, being presumably translocated to the seeds. A similar temporary storage in the hull of nitrogen subsequently transferred to the seeds has been noted by other workers (Pfenninger, 1909; Schellenberg, 1916; Bisson & Jones, 1932; McKee, Robertson, & Lee, 1955). The hull also acts as a reservoir for carbohydrate. The leaves probably supplied most of the nitrogen moving to the seeds from other parts of the plant. The total amount lost from leaves and hulls was much less than that gained by the seeds. The total nitrogen of the plant was trebled, by

uptake from roots or root-nodules, after fruiting began (Table 13). These results, where comparable, agree reasonably well with those of Anderson (1866b), except that in his experiment the stems accumulated more nitrogen early in the season and released some of it later.

The distribution of nitrogen between different parts of the tobacco plant throughout its life-history has been recorded by Vickery, Pucher, Leavenworth, & Wakeman (1935) and by Vlădescu (1938a, b, c). The roots at all stages contained less than 10 per cent of the total nitrogen. In young plants a very high proportion (80 to 90 per cent) was in the leaves. The stem had 20 to 25 per cent at all stages except the earliest in Vlădescu's work; Vickery and his co-workers reported much greater variation. The fruits contained less than half the nitrogen of the mature plant, a contrast with the bean. The transport of soluble nitrogenous material to developing fruits has been shown also for maize (Hornberger & Von Raumer, 1882; Hay, Earley, & De Turk, 1953), cotton (Maskell & Mason, 1930), and barley (Deleano & Gotterbarm, 1936). In maize, about 70 per cent of the total nitrogen of the plant is concentrated in the mature grain. Hay *et al.* (1953) found that 40 per cent of this nitrogen came from the roots (or the soil) after pollination. The leaves supplied 60 per cent of the nitrogen translocated to the seeds from above-ground parts of the plant, the stem 28 per cent, and the husk, which appears to be physiologically though not morphologically analogous to the hull of the bean, 12 per cent. Urea supplied through the leaves of wheat plants at the time of flowering increased the protein content of the grain; the greatest increase in total yield was obtained by spraying a few weeks before flowering (Reeves, 1954).

Deleano & Bordeianu (1933) showed that in the horse chestnut (*Aesculus hippocastanum*) the leaves returned a large part of their nitrogen to the branches during the autumn; over the same period a rapid increase occurred in the nitrogen content of the developing fruits, which probably drew their supplies in part from the senescent leaves. Gaumann (1935) found that in the beech (*Fagus sylvatica*) leaf formation in the spring required five times as much nitrogen as was used later in the season to form flowers and fruits. A lower rate of return from the leaves than that actually observed in this species would thus be fully adequate to cover the nitrogen requirements for fruiting. Figures for several deciduous fruit trees (Van Slyke, Taylor, & Andrews, 1905) suggest that for an individual tree mature but not senescent leaves contain amounts of nitrogen comparable to that lost in the fruit crop. Assuming that 80 per cent of the nitrogen of senescent leaves returns

to the tree, more than twice the amount used in fruit formation would be available from this source in the peach trees studied. Apples and pears showed on this basis a slight excess of available nitrogen from the leaves; plums and quinces lost slightly more nitrogen in the fruit than could be supplied from senescent leaves. The outlay of phosphorus and potassium in the fruit crop of these trees considerably exceeds the amount recoverable from the senescent leaves, even assuming a high rate of return for these elements. The contents of nitrogen and other elements reported for the leaves in this work are probably minimum estimates. Leaves were sampled for analysis at a stage when they "showed a tendency to drop" and might already have returned to the trunk some of their mobile constituents.

The data of Berthelot & André (1891) (Table 15) for the distribution of sulphur in *Sinapis alba* at successive stages of development show a picture very similar to that outlined above for nitrogen. There is a

TABLE 15

*Distribution of sulphur in developing plants of Sinapis alba*  
(Calculated from data of Berthelot & André, 1891.)

	21 May		1 June		24 June		15 July	
	(Before flowering)		(Beginning of flowering)		(End of flowering)		(Fruiting)	
	mg S per plant	Per cent of total S	mg S per plant	Per cent of total S	mg S per plant	Per cent of total S	mg S per plant	Per cent of total S
Roots	0.3	12	5.8	34	2.2	3	1.2	2
Stems	1.3	52	4.4	25	34.3	41	11.8	18
Leaves	0.9	36	5.3	31	24.5	29	9.0	14
Inflorescences			1.8	10	23.4	27	42.1	66
TOTAL	2.5		17.3		84.4		64.1	

Seeds at planting on 15 April contained 0.02 mg S; seedlings on 12 May contained 0.4 mg S.

rapid increase in absolute and relative amounts of sulphur in the inflorescence and the fruits formed from it. The sulphur transferred to the fruits comes largely from the stem, which is a temporary storage organ. The sulphur of the leaves decreases sharply in the later stages; some may be lost by leaf-fall, as the total sulphur of the plant falls at this time.

### C. Compounds found in conducting tissues

There has been much controversy regarding the relative importance of phloem and xylem as conducting tissues for organic and inorganic

substances. Here it need only be said that in both tissues the occurrence of conduction seems to be well established in some species and under some conditions. The nature of the compounds found in both phloem and xylem is, therefore, relevant in considering the phenomena of conduction.

Several authors (Dixon, 1933; Moose, 1938; Ziegler, 1956) found phloem sap to contain much sucrose and little organic nitrogen, though amino-acids were present. Mittler (1953) detected by paper chromatography asparagine, glutamine, and 10 other amino-acids in the phloem sap from stems of willow (*Salix*) at seasons when a high rate of transport to or from the leaves might be expected, i.e. when the leaves were either actively growing or senescent. Phloem sap from stems bearing mature leaves contained only traces of asparagine, glutamine, and the corresponding dicarboxylic amino-acids. Ziegler (1956) found in the phloem sap of *Acer platanoides* and *Quercus* spp. larger amounts of amino-acids, especially aspartic acid and glutamic acid, in autumn when leaf-fall was approaching than in summer when the leaves were mature but not senescent. Phloem sap of the vine (*Vitis vinifera*) contains relatively large amounts of citrulline (Meyer-Mevius, 1959).

Nitrogenous compounds also occur in the xylem sap. Anderssen (1929) recorded appreciable amounts of amino and amide nitrogen, together with traces of nitrate, in the xylem sap of pear and apricot trees. Bollard (1953a, b, 1957a) found that in apple trees this sap contained 10  $\mu\text{g}$  N/ml during the winter, increasing to 20  $\mu\text{g}$  three weeks before flowering and to 150  $\mu\text{g}$  for three weeks at flowering time. The concentration then gradually declined and by early autumn had returned to the minimum level. The main soluble nitrogenous compounds present were asparagine, glutamine, aspartic acid, and glutamic acid; other amino-acids and probably peptides were also detected. Surveys covering numerous species (Möthes & Engelbrecht, 1952b; Reuter, 1957a; Bollard, 1957b, c) have shown a rather wide range of nitrogenous compounds to be important constituents of the xylem sap, particularly in spring, in different woody species. Such compounds include  $\delta$ -N-acetylornithine, alanine, allantoin, allantoic acid, asparagine, aspartic acid,  $\gamma$ -aminobutyric acid, arginine, azetidine-2-carboxylic acid, citrulline, glutamic acid, glutamine, leucine, phenylalanine, serine, and valine. The importance of the glyoxylic ureides, allantoin, and allantoic acid, as mobile forms of nitrogen is indicated by the high proportion of the total soluble nitrogen which they represent in some species, e.g. *Acer pseudoplatanus* and *Wistaria sinensis* (Brunel & Échevin, 1938;

Échevin, Brunel, & Sartorius, 1940). Peptides are also recorded, e.g. in *Acer saccharum* (Pollard & Sproston, 1954) and in maize (Fejér & Kónya, 1958).

Some data are available on nitrogenous constituents of the xylem sap in herbaceous and semi-woody plants. Nitrate is recorded in significant amounts in some species, e.g. cotton (*Gossypium*) (Mason & Maskell, 1931) and various grasses (Pierro & Pohlman, 1933). It occurs also in xylem sap of some woody species, e.g. *Pandanus titchii* (Sideris, Krauss, & Young, 1937) and the vine (*Vitis vinifera*), where Wormall (1924) found almost all the nitrogen to consist of nitrate plus small amounts of nitrite. In the peanut (*Arachis hypogaea*) the main nitrogenous constituent of the sap is  $\gamma$ -methyleneglutamine (Fowden, 1954a); in the pumpkin (*Cucurbita pepo*) numerous amino-acids are found, the most important being alanine,  $\gamma$ -aminobutyric acid, and glutamic acid (Kulayeva, Silina, & Kursanov, 1957). Nitrate, however, represents 80 per cent of the total soluble nitrogen (Kretovich, Yevstignoyova, Aseyeva, & Savkina, 1959). In cucumber and tomato (Van Die, 1958, 1959) glutamine is the dominant nitrogenous compound. In these species the sap contained much pyruvic acid and  $\alpha$ -ketoglutaric acid, reducing sugars being almost absent; xylem sap of pumpkin also contains abundant pyruvic acid (Kursanov & Kulayeva, 1957). Van Die (1959) recorded a large diurnal variation in the amino-acid content of the xylem sap in tomato plants grown in strictly controlled environments. The causes of this rhythm are obscure, but it suggests that the substances affected are active metabolites. Variations due to external conditions are probably superimposed on such endogenous rhythms in natural conditions. Combes, Brunel, & Chabert (1942a) cultivated plants of *Veronica anagallis* at several light intensities. Amides predominated in the soluble nitrogen of plants grown in full sunlight, but were largely replaced by nitrate at low light intensities. At intermediate levels of illumination, both nitrate and amides were found. Nitrate disappeared at the beginning of flowering, except at very low light intensities.

In barley, tomato, sunflower, bean, and willow, phosphorus moves in the xylem sap partly as inorganic phosphate and partly in organic combination (Tolbert & Wiehe, 1955). The organic phosphorus compounds were not identified, they were neither phospholipids nor sugar phosphates. Sulphate seemed to be the only mobile form of sulphur. Fejér (1957, 1958), however, detected methionine and glutathione in bleeding sap of maize, especially at the start of active growth, at

flowering, and while the grain was ripening. In sugar beet methionine moves from the roots to the shoot (Vlasyuk, Kosmatyi, & Klimovitskaya, 1957). Renter (1957c) showed that in bleeding sap of *Nicotiana rustica* glutamino and asparagino, prominent at most stages of development, were overshadowed at flowering by alanine,  $\gamma$ -aminobutyric acid, and proline, which at other times were minor constituents. Various workers, e.g. Dawson (1942b), Hieko (1942), and Wada, Kisaki & Ihida (1959) found alkaloids in bleeding sap, thus providing a link in the chain of evidence for the root as a major site of alkaloid synthesis.

Enzymes may pass from one part of the plant to another, though transport of protein as such is not clearly established. Sisakyan & Kobyakova (1951) suggested that enzymes (invertase, phosphorylase, phosphoglucomutase) moved to new leaves on sprouting sugar-beet roots, and from senescent leaves to the roots in autumn. These conclusions are consistent with the changes reported in enzymatic activity in different organs of the plant during development. Enzymatic proteins may, however, be hydrolysed and the breakdown products translocated for resynthesis elsewhere.

## CHAPTER 15

# THE CYCLE OF NITROGEN IN NATURE

### A. Geochemistry of nitrogen

All living matter known to us contains nitrogen. Very numerous nitrogen compounds are recorded in organisms, and the true total must be much greater. All living species (the number now may be of the order of  $10^6$ ) probably form distinctive proteins and nucleic acids, and perhaps other special nitrogen compounds. The chemical versatility of nitrogen is further emphasized by a vast array of synthetic compounds prepared in the last hundred years. The reactivity of nitrogen compounds contrasts with the chemical inertness of the free gas. It is not clear why the gas is so inert. The nitrogen molecule is generally held to contain a triple bond. This might be expected to be unstable and reactive, but one of the most stable bonds that nitrogen atoms enter is that linking them in pairs as the unreactive molecule of the free gas.

Most of the earth's nitrogen occurs (Redfield, 1958) in the atmosphere, which has roughly  $3.8 \times 10^{21}$  g ( $3.7 \times 10^{15}$  long tons) of the element; sedimentary rocks contain rather more than one-tenth of this amount, probably arising largely from organic materials deposited in them; the ocean contains  $2 \times 10^{19}$  g of dissolved nitrogen and, of greater importance for marine plants,  $7 \times 10^7$  g of nitrate nitrogen. Most of the nitrate is in deep water; near the surface it may be almost completely assimilated by plankton. Deep nitrate-rich water wells up in certain parts of the ocean; surface currents also tend to equalize the concentration in different areas.

The origin of the nitrogen of rocks is uncertain. In sedimentary rocks it is often supposed to arise essentially from organic remains, but Stevenson (1959) reported that in both shale and granite half of the total nitrogen was held in the lattice structure of silicate minerals as ammonium ions, which he considered an original constituent of the mineral rather than a casual replacement for some other ion. Abelson (1954b) reported briefly the isolation of alanine, glutamic acid, and valine from Ordovician and Jurassic fossils. Lehmann & Prashmowsky



(1959), in studies which they described as palaeobiogeochemical, detected a considerable range of amino-acids in fossils dating from the Lower Devonian to the Tertiary, or in the rocky matrix surrounding them. Arginine, aspartic acid, asparagine, glutamic acid, histidine, and lysine were found regularly; alanine, glycine, isoleucine, leucine, and valine occurred sporadically; proline, serine, threonine, tyrosine, tryptophan, and the sulphur-containing amino-acids were rare. The amino-acid content decreased with the distance from a fossil into the surrounding rock, but the acids present and their proportions were unchanged. It is possible that, as stated by the authors, these amino-acids arose from the tissues of fossilized organisms; a later absorption of amino-acids from decaying organic matter seems, however, not to be entirely excluded. Heijenskjöld & Möllerberg (1958) obtained aspartic acid, glutamic acid and glycine from hydrolysates of anthracite estimated to be 250 million years old.

## B. Nitrogenous compounds in the atmosphere

The presence of nitrate in rain and snow, reported by Marggraf (1761-67), was confirmed by Bergman (1788-90) and many later workers, e.g. Jones (1851). De Saussure (1804) showed that the atmosphere contained ammonia, which was detected in sea water by Marcet (1822). Attention was focussed on atmospheric ammonia by the claim of Liebig (1843) that it was the main source of nitrogen for plants. Work at Rothamsted (Way, 1855, 1856; Lawes, Gilbert, & Warington, 1881) and in France (Barral, 1852*a, b*; Bineau, 1852; Boussingault, 1854, 1858) showed that less ammonia was available in this way than Liebig supposed, and provided much information on the amounts of ammonia and nitrate reaching the ground in rain. Combined nitrogen occurs in the atmosphere only in small and variable amounts; it is, nevertheless, more directly relevant to problems of plant nutrition than the great inert mass of atmospheric molecular nitrogen. Several workers (Way, 1855; Miller, 1905; Russell & Richards, 1919; Eriksson, 1952) have reviewed the large body of recorded data on nitrogen compounds in the atmosphere and in atmospheric precipitation, the latter referring usually to rain but including also snow, hail, dew, fog, and hoarfrost. Less extensive data are available for various other elements occurring in gaseous or particulate form in the atmosphere, e.g. chlorine (Barral, 1852*a*; Anderson, 1915, 1945; Harrison & Williams, 1897; Kinch, 1900; Wood & Wilsmore, 1929; Teakle, 1937), sulphur (Gray, 1888; Bertrand,

1935, Alway, Marsh, & Methley, 1937, Bertramson, Fried, & Tisdale, 1950), bromine and iodine (Marchand, 1852, Chatin, 1853, Cauet, 1937), calcium and magnesium (Tarcy, 1931, Bertrand, 1943), potassium (Anderson, 1945, Bertrand, 1945), and arsenic (Xhoris, 1945). Arsenic, and in part sulphur, are attributable to atmospheric pollution by human activities, most of the other elements listed reach the atmosphere mainly from the sea.

At Rothamsted over the period 1888–1916 (Russell & Richards, 1919) the average amount of nitrogen reaching the soil as ammonia was 2.64 lb/acre/year (2.96 kg/ha/year), almost exactly half this amount was received as nitrate. The rain contained on the average 0.4 p.p.m. of nitrogen as ammonia and 0.2 p.p.m. as nitrate. In cities with marked atmospheric pollution, such as London or Newcastle on Tyne, the ammonia content of the rain was higher by a factor of about six, nitrate was much less affected. The total nitrogen reaching the soil per unit area tends to increase with the annual rainfall, indicating that the concentration of combined nitrogen in rain is independent of the total rainfall. The amount of nitrogen reaching the soil as nitrate and ammonium lies usually between 2 and 10 kg/ha/year in Europe, figures in this range are recorded for other parts of the world, but observations are comparatively few. There are suggestions in both the northern (Ångström & Höghberg, 1952) and southern (Anderson, 1915) hemispheres of a higher combined nitrogen content in tropical than in polar air. Snow appears to scrub nitrogenous compounds from the atmosphere less efficiently than rain (Shutt, 1908, Herman & Gorham, 1957).

Nitrite occurs in rain, but its concentration is low compared with that of nitrate (Hudig, 1912, Anderson, 1915, Drover & Barrett-Lennard, 1950, Meyer & Pampfer, 1959).

Several observers have found appreciable amounts of organically combined nitrogen (usually cited as albuminoid N) in rain. Tissandier (1875) detected organic matter in snow collected in Paris. Berthelot & André (1887a) found amino nitrogen to represent up to 75 per cent of the total nitrogen in rain collected at Meudon (France). At Rothamsted, organic nitrogen in the rain almost exactly equalled nitrate nitrogen (Miller, 1905). Rain collected at Lincoln, New Zealand contained variable amounts of organic nitrogen but always considerably less than that present as nitrate (Gray, 1888). The high figure of 5.4 lb organic N/acre/year (6.05 kg/ha/year) is reported for Sylhet, India (Das, Sen, & Pal, 1933); this represents 65 per cent of the total nitrogen. The large total amount of nitrogen may be correlated with the high rainfall at

Sylhet—155 inches (3,950 mm) in the year when the analyses were made. Wilson (1959*a, b*) found that snow collected in New Zealand at altitudes between 4,000 and 8,000 feet (1,200 to 2,400 m) had a large part (up to 90 per cent) of its nitrogen in organic combination. Free amino-acids occur in minute amounts in rain (Fonselius, 1954) and in the atmosphere (Munczak, 1960).

## C. Origin of the combined nitrogen of the atmosphere

### (a) SOURCES OF ATMOSPHERIC NITRATE

Way (1855) remarked that after the demonstration (Cavendish, 1785) of nitric acid formation by electric sparks acting on a mixture of nitrogen and oxygen, it became usual to attribute a similar origin to the nitrate found in rain. This view is still popular; its chief defect is that, although lightning and perhaps silent electrical discharges may be supposed to form some nitrate in the upper air, no clear correlation appears to exist between the amount of nitrate carried down in the rain at a particular place and the number or intensity of thunderstorms there. An alternative source of nitrate is the photochemical oxidation by ultra-violet radiation of ammonia (or even of nitrogen) to nitric oxide. This possibility has been discussed for some time but little firm evidence for or against it has been produced. Oxidation of ammonia to nitrate would affect only the proportions of two forms of combined nitrogen without altering their total amount; any oxidation of nitrogen would, of course, increase the supply of combined nitrogen.

Lewis & Randall (1923) pointed out that, although the reaction proceeds at an insignificant speed in standard conditions, the formation of nitric acid from its elements involves a decrease in free energy. This reaction, if equilibrium were attained, would remove all oxygen from the atmosphere and convert the sea and other terrestrial waters to a dilute solution of nitric acid. They expressed the hope that no natural catalyst for the reaction will appear. No direct biological oxidation of nitrogen has been established, though it has been postulated by some workers on nitrogen fixation. Nitrate, however, arises indirectly from gaseous nitrogen by nitrification of ammonia or organic nitrogenous compounds formed by nitrogen-fixing organisms. Nitrogen fixers and nitrifiers working in succession are thus equivalent to a "natural catalyst". Since their activities are counterbalanced by biological nitrate reduction and denitrification, no net accumulation of nitrate occurs on a world scale.

## (b) SOURCES OF ATMOSPHERIC AMMONIA

Ammonia reaches the atmosphere in several ways whose occurrence is reasonably well established though much uncertainty persists regarding their quantitative importance. Schloessing (1875*a, b, c*, 1876) considered the ocean as a reservoir of ammonia which diffused to the atmosphere and was transported by winds to the continents, where it was absorbed by soil, or directly by plants, as well as being washed down by rain. His estimate for the rate of ammonia absorption by the soil seems improbably high (40 kg N/ha/year: 36 lb/acre/year); even higher values are, however, suggested by Ingham (1950*a, b*).

Muntz & Aubin (1882) analysed air collected at 2,900 m (9,500 feet) on the Pic du Midi and presumably uncontaminated. It contained an average of 13  $\mu\text{g/litre}$  of ammonia. Lévy (1880) found about double this amount as the average value for a series of analyses made throughout the year at Montsouris (France). These values are small but appear (Eriksson, 1952) considerably higher than the equilibrium value calculated from the ammonia content of the sea. If the sea is the main source of atmospheric ammonia, diffusion cannot be the main means of transfer. Another possibility is spray, which is known to be carried inland for long distances and to transport large amounts of soluble salts, which accumulate in arid areas. Lemery (1693), observing that although rivers continuously carry dissolved salts to the sea, its salt content does not appear to increase, concluded that some process must return salt from the sea to the land. This process he found in the transport inland of spray and the deposition of its salt on the ground. More recent workers (e.g. Wood & Wilshire, 1929; Anderson, 1945) have clearly shown that important amounts of chloride are transported in this way even for hundreds of miles inland. If the spray has the same composition as sea water in bulk, it could carry only insignificant amounts of ammonia. There is, however, some evidence that in the sea ammonia is adsorbed to particulate matter which tends to concentrate at the surface (Cooper, 1948); a comparatively high concentration of ammonia has also been observed in the surface layer of lake water (Karcher, 1939). Whatever the relative contributions of spray and of diffusion may be, the sea can hardly be a major source of atmospheric ammonia as the ammonia content of rain in seaside localities is generally low. Miller (1913) found the ammonia content of rain collected close to the sea in the Hebrides and Iceland, mostly at lighthouses, to be low compared with samples from other British localities with little atmospheric pollution.

The decay of organic residues must yield large amounts of ammonia, but comparatively little of this can reach the atmosphere. Much of the decomposition occurs in soil or in water, where gaseous ammonia is likely to be absorbed. This source no doubt supplies some atmospheric ammonia; its quantitative importance is difficult to assess, but unlikely to be large. Plants are known (Klein & Steiner, 1928; Steiner & Löffler, 1931) to give off small amounts of gaseous ammonia from their leaves and flowers. This continuous source may be more important than is generally recognized.

It is possible that in natural conditions, particularly in dense vegetation, ammonia is largely reabsorbed by plants or by the soil instead of reaching the general store in the atmosphere. Berthelot & André (1887b), however, observed a constant emission of ammonia from grass-covered soil. The respiration of animals may also contribute some gaseous ammonia. The subject has been studied over a long period, but no clear picture of the amounts involved has emerged. Marchand (1844) stated, without experimental data, that the frog produced gaseous ammonia. Regnault & Reiset (1849), in an elaborate report on very careful studies of respiration in the dog, rabbit, and fowl, recorded a consistent but very small output of ammonia. Lossen (1865) and Ransome (1870) confirmed this in man, though with reservations as to its metabolic significance; decaying food residues in the mouth and carious teeth were suggested as possible sources. The matter was taken up again by Robin, Travis, Bromberg, Forkner, & Tyler (1959), who concluded that the mammalian lung excretes only very minute amounts of ammonia, and these irregularly.

The main source of ammonia in the atmosphere is probably combustion of organic matter. Its importance is suggested by the high ammonia content, arising largely from the burning of coal, of the rainfall in industrial regions, and also by the substantial amounts of ammonia recovered from coal burnt in gas retorts and coke ovens. Black coal contains about 2 per cent of nitrogen; lignite about 1 per cent (Ramachandran, Mukherjee, & Lahiri, 1959). In regions where dried dung is used as fuel its nitrogen must supply appreciable amounts of ammonia to the air. Kishen (1959) estimated that 65 million tons of air-dry dung are burnt annually in India. Forest fires are another source for which little quantitative information is available; Shutt (1915) recorded a high ammonia content in the air at Ottawa, Canada, after forest fires.

Volcanic activity also releases ammonia to the atmosphere. The

effects may be locally important, but are probably small at present on a world scale. Shipley (1919b) found in Alaska that near fumaroles the rain had much more ammonia than that collected a short distance away. Remarkably high concentrations of ammonium ion (500 to 700 p.p.m.) are recorded for hot springs in New Zealand (Wilson, 1953) and North America (White, Sandberg, & Brannock, 1953). Volcanic ammonia may not all be a net addition to the combined nitrogen available for biological activity. It may arise in part from combined nitrogen of organic origin contained in rocks near the volcano. Smoke from slowly burning vegetable debris can deposit crystalline ammonium chloride (Hartung & Rivett, 1915).

Combustion of organic materials, mainly through deliberate human activity but with some contribution from forest fires, is probably the largest single source of atmospheric ammonia. Ammonia reaches the atmosphere in this way as a final stage in the decomposition of organic matter varying in age from current active tissue in forest fires to long-fossilized plant residues in coal. Burning of coal returns to the atmosphere, in a readily available form, nitrogen absorbed by plants in earlier geological epochs.

#### (e) SOURCES OF ORGANIC NITROGEN IN THE ATMOSPHERE

A substantial part of the total nitrogen in rain may be in organic form. Much of the organic nitrogen of the atmosphere is in small particles such as pollen, spores, bacteria, and dust carried from the earth's surface by ascending currents. Wilson (1959a, b) found in New Zealand that snow at altitudes between 5,000 and 8,000 feet (1,500 to 2,400 m) had up to 80 per cent of its total nitrogen in organic combination. The remaining nitrogen was almost entirely in ammonia; nitrate was low or absent. The snow was sampled at a season when contamination by plant and animal debris was considered unlikely. This assumption may not have been entirely correct; such particles travel over great distances in the wind, but they probably did not account for much of the organic nitrogen present. The ocean was accordingly suggested as the main source of the organic nitrogen. The transport inland of sodium chloride in spray particles carried by the wind has long been recognized. Wilson's new contribution is to suggest as the source of spray a thin surface layer differing greatly in composition from the bulk of the ocean. This layer is assumed to contain planktonic debris which, being lighter than sea water, accumulates at the surface and contains a much higher concentration of organic nitrogenous material than the ocean as a whole. It might also reasonably

be assumed to be enriched in potassium (accumulated by planktonic organisms) and in ammonia. There is some other evidence for an accumulation of ammonia in the surface layer of the sea (Cooper, 1948) and of fresh water (Karcher, 1939). These suggestions are consistent with the observations (Wilson, 1959a, b) that the snow samples had higher potassium/sodium and ammonium/nitrate ratios than would be expected from analyses of sea water in bulk. This process may continuously transfer nitrogen and other nutrients from sea to land.

#### D. Transformation of nitrogen in the sea

Rain falling on the sea contains ammonia and nitrate. These compounds and also organic debris are carried down in rivers. Nitrogen fixation by marine bacteria and blue-green algae is sometimes stated to be a major factor in the nitrogen economy of the sea, but this assumption is not supported by much direct evidence. *Azotobacter* and nitrogen-fixing species of *Clostridium* occur in shallow water, massed on the surface of other organisms or living in bottom mud. The supply of organic matter is likely to limit their activity in the open sea, though a surface layer of the type envisaged by Wilson (1959b) would be more favourable than sea water in bulk. Photosynthetic blue-green algae seem more promising as planktonic nitrogen-fixers, but little is known of the efficiency of marine species in this respect.

The nitrogenous constituents of dead marine plants and animals, and of other organic remains reaching the sea, break down with the formation of ammonia; urea, amino-acids, and amines probably occur as transient intermediates. Ammonia may be utilized directly by phytoplankton; it can also be oxidized to nitrite and nitrate, both known to be constituents of sea water. Hyponitrite is a plausible intermediate; there is evidence (Cooper, 1938) for its occurrence in the sea. Hydroxylamine, another likely intermediate, would be unstable in sea water, which is alkaline (pH 8); it has, however, been detected in a fresh-water lake (Tanaka, 1953). In this case hydroxylamine appears to have been an intermediate in the bacterial reduction of nitrate; it can equally arise in the reverse process, nitrification of ammonia. These transformations of nitrate and ammonia do not affect the total amount of combined nitrogen, but it is reduced by bacterial denitrification. This occurs in the sea (Gran, 1901) and in lakes (Klein & Steiner, 1929), but seems unlikely to be a major factor in the nitrogen economy of the sea. A much more substantial withdrawal of combined nitrogen from biological circulation results from the continuous rain of animal

remains upon the sea floor. These are buried in sediments and presumably account for the comparatively high nitrogen content of sedimentary rocks. Nitrogen concentrated in the bodies of marine animals, obtained directly or indirectly from phytoplankton and so from the reserves of combined inorganic nitrogen in the sea, is thus diverted to a situation where for geologically long periods it takes no part in biological transformations. Bacteria exist on the bottom at great depths, but their activities are clearly insufficient to release all the nitrogen of the sediments, though they may contribute to the reserve of nitrate in deep ocean waters.

It is customary to cite the average nitrogen content of eruptive rocks as 50 p.p.m. and that of sedimentary rocks as 500 p.p.m. Actual values vary widely, Hall & Miller (1908) report figures below 100 p.p.m. for sandstones and over 1,000 p.p.m. for shales. There is no doubt, however, of the generally high nitrogen content of sedimentary rocks. Some poor soils developed from sandstone may derive a substantial part of their nitrogen from the parent rock, as on the Hawkesbury Sandstone in the Sydney district (Hannon, 1956). This rock contains about 200 p.p.m. of nitrogen and the soils derived from it 300 to 600 p.p.m. Cretaceous and Tertiary shales and sandstones in the Book Cliffs (Utah-Wyoming) and Tecopa (California) districts contain very large total amounts of nitrate, probably much more than the nitrate deposits of Chile, but the concentration is nowhere high enough for profitable exploitation (Free, 1912; Stewart & Peterson, 1914). Some nitrogen once buried on the sea floor is thus released for further use by plants after the long cycle of geological uplift and erosion, but the amounts so liberated are probably negligible compared with the inaccessible store in the sediments of the ocean bed.

#### E. The nitrogen cycle on land

Higher plants in general draw their nitrogen supplies from nitrogenous compounds in the soil. The combined nitrogen of the soil has four main sources: (i) combined nitrogen is released, perhaps with secondary transformations, from the parent rock, (ii) rain brings nitrate and ammonia, gaseous ammonia may also be absorbed directly from the air, (iii) organic matter (leaf litter, animal bodies and excreta) falling on the soil is broken down by microorganisms and its nitrogenous constituents converted to soluble compounds assimilable by plant roots, (iv) free nitrogen is fixed by free living and symbiotic microorganisms. Nitrogen so fixed is largely incorporated into the



and Wyoming were unusually rich in nitrate (1 to 10 tons/nere-foot = 0.05 to 0.5 lb/cubic foot = 0.8 to 8 kg/enbie metre). They attributed the accumulation of nitrate reported by Headden (1910, 1911, 1914) to its concentration in the surface soil after moving upwards in solution from the underlying rock. This theory, though not clearly explaining the occurrence of high-nitrate soils in small well-defined areas, seems more plausible than the assumption of locally very intense fixation.

Symbiotic fixation can add substantial amounts of nitrogen to the soil under pastures well stocked with vigorous plants of adequately nodulated legumes. Both legumes and other nodulated plants appear to play a major part in the nitrogen economy of some natural communities. For other communities, such as tropical rain-forest, information is scanty and somewhat contradictory. In undisturbed rain-forest there may be an almost closed local cycle of nitrogen, the amount reaching the soil in leaf litter being in approximate equilibrium with that taken up by plant roots. The very low wind velocities at ground level within such forests would permit the re-absorption by plants of any gaseous ammonia given off by the soil, and the layer of slowly decaying litter on the ground would reduce losses of nitrogenous materials by erosion and leaching. In such conditions of temporary equilibrium the soil might contain enough available nitrogen to depress the formation and activity of nitrogen-fixing nodules. If this picture is correct, the nodules of leguminous forest trees provide a regulatory mechanism capable of restoring nitrogen lost when the equilibrium is disturbed, or of improving the nitrogen status of newly developed communities, but not very active in well-established forest. This would be consistent with observations (Bonnier, 1957; Bonnier & Seeger, 1958) that in tropical forest leguminous trees may lack nodules though potentially capable of forming them.

Combined nitrogen is lost from the soil in several ways. Bacterial denitrification occurs but its quantitative importance is uncertain. The main losses are probably by erosion and leaching of the soil, which in part redistribute combined nitrogen over the surface of the land, but finally transport it to the sea, representing for practical purposes a permanent net loss to land vegetation. Erosion and leaching may not remove much nitrogen each year from the soil below closed and stable plant communities; their importance is much greater in open communities and on soils disturbed in any way. It is probable that transfer of nitrogen from land to sea exceeds the amount moving by various agencies in the reverse direction.

## F. Effect of human activities on the nitrogen cycle

Agriculture is a major interference with the vegetation. The precise place and date of its invention are unknown, but that in the last ten thousand years or so it has spread over land surface of the earth, profoundly modifying soils and plant communities. Cultivated land differs from virgin country in many ways; an important aspect is that removal of crops represents an export of elements, including nitrogen. In a stable natural plant community the net annual loss of nitrogen, as we have seen, may be small. A crop, such as wheat, removes substantial amounts of nitrogen from the soil in each growing season. In Australian conditions, wheat yields and protein content of wheat are particularly high, but this should be deducted 1 lb N/acre supplied in the seed, 3 lb N/acre received in rainfall. The allowance for nitrogen in rainfall should be doubled if wheat crops alternate with fallow; the amount removed then becomes 23 lb N/acre/crop, or 11.5 lb N/acre/year (13 kg/ha/year). This loss may be compensated in part through the contribution of legumes during the fallow year; non-symbiotic nitrogen fixation is small but in Australian wheat-belt conditions it is small. The most probable result is a gradual improvement of the soil in nitrogen even when crop yields are comparatively high, of course, accelerate the process. The general principle is similar for other cereals, except rice, which is grown in flooded soils where fixation of nitrogen by blue-green algae may be important. The drain of nitrogen from the soil will be less with pulses and leguminous crops; their cultivation may even improve the status of the soil. This is not, however, necessarily the case. Cereals removed in the crop, contain most of the nitrogen of the plant; the general is drawn both from the soil via the roots and from the root-nodules.

Grazing also removes large amounts of nitrogen from the soil in such products as milk, wool, and the bodies of stock sold. When practised on pastures with a good content of legume, the return of nitrogen through fixation is much greater than with cereal plants, and may provide an excess available to crops if the land is ploughed up. On intensively managed pastures large amounts of nitrogen are returned to the soil in animal excreta; these include urea and uric acid, both known to be good nitrogen sources.

Addition of superphosphate to a small fresh-water lake (Einsle, 1941) led to a substantial increase in its total nitrogen content, presumably through the increased activity of nitrogen-fixing bacteria or blue-green algae. The effect appears analogous to that occurring on land when legume-containing pastures are fertilized with superphosphate.

The methods now considered desirable for the disposal of human excreta transfer large amounts of nitrogen and other plant nutrients from land to sea. Human manure is, of course, a familiar fertilizer in many countries; the traditional methods of application are, however, suspect from the point of view of public health. Alternative methods avoiding losses to the sea without spreading pathogenic organisms are possible and may well be adopted in the future. In the meantime, fishing obtains from the sea substantial amounts of human food, thus recovering as protein a part of the nitrogen leaving the land in forms less suitable for human food. Losses of combined nitrogen large enough to be a serious drain on the agricultural capital of the land would have only a marginal effect on available nitrogen in the sea, and cannot be condoned as a transfer from one productive area to another. Some areas are already over-fished, but the total production of marine foods could probably be much increased.

No land animal other than man recovers much nitrogen from the sea, but gregarious fish-eating birds deposit it in large amounts in droppings which gave rise to guano and probably to the very important rock phosphate deposits of Nauru, Ocean Island, and Christmas Island (Indian Ocean). If rock phosphate arises from nitrogenous organic material, nitrogen is presumably lost by leaching or volatilized as ammonia or ammonium carbonate. A marine origin is possible for the nitrate deposits of Chile, which occur in almost rainless areas and would be dissipated by even moderate rainfall. Their origin has been much disputed without any explanation being generally accepted. Müntz & Marciano (1885) and Müntz (1887a) suggested that accumulations of organic matter (excreta of sea birds, or fish killed in some catastrophe) formed ammonia which by bacterial action led to calcium nitrate, converted to sodium nitrate by double decomposition during an incursion of sea water. The iodate (Lembert, 1843), and bromate associated with the nitrate were attributed to biological oxidation of iodide and bromide. During microbiological nitrification iodide is oxidized (Müntz, 1885) to iodate, now recognized (Sugawara, 1955) as containing most of the iodine in sea water.

The low phosphate content of the nitrate deposits requires explanation if they arose from animal matter. The nitrogen/phosphorus ratio presumably varies from species to species but the range of variation may not be great. In man it is close to 3 (Mitchell Hamilton Steggerda & Bean 1945) and similar values are reported for fish. Leaching would remove ammonia or nitrate before phosphate. Nitrates might be transported in ground water and deposited at the surface by evaporation in dry areas. This would explain their separation from phosphate but not the complete disappearance of the latter. Plant tissues have a much higher nitrogen/phosphorus ratio (15 or above) but seem an unpromising raw material owing to their low nitrogen content. An atmospheric origin for the nitrogen of the nitrate beds would simplify the problem in some ways but implies an intensity of fixation unknown elsewhere except perhaps in the peculiar conditions reported for some Colorado soils (Headden 1914).

Human activities affect the nitrogen cycle at many points. Industrial fixation of atmospheric nitrogen and the widespread use of nitrate formerly locked up in waterless South American deserts increase the supply of combined nitrogen in agricultural land. Phosphatic fertilizers fortified in some areas with molybdenum and other micronutrients increase fixation by cultivated legumes. Their phosphorus probably comes ultimately from the sea passing through plankton and fish before accumulating in sea bird droppings, the source of phosphate deposits. Selection of efficient rhizobial strains is another important means of encouraging symbiotic fixation. Against these positive effects must be set increased losses of combined nitrogen by leaching and erosion which may in part be inherent in agricultural and forestry practice but are often far above the unavoidable minimum rates. No accurate estimate of the net effect of these contrasting processes is possible; the available data are hardly adequate to establish with certainty whether the land is losing nitrogen on balance. It seems likely that losses to the sea exceed accretions from the atmosphere plus amounts returned from the sea but this is not firmly established.

### G Nitrogen supplies and human food

It is usual in studying nutritional problems to state human requirements for nitrogenous materials as grams of protein per day. Many of the essential vitamins also contain nitrogen but the actual amount of the element required for an adequate supply of vitamins is very small. Protein *per se* may not be an essential feature of the human diet being

replacable by mixtures of about ten of the twenty common protein amino-acids. Experiments with animals (Woolley, 1945; Womack & Rose, 1946; Maddy & Elvehjem, 1949; Benton, Spivey, & Elvehjem, 1957) suggest that proteins give somewhat higher growth rates than can be achieved with mixtures of amino-acids. It is not clear whether this stimulation should be attributed to the availability in protein of useful pre-formed peptides or of other substances, not necessarily amino-acids, contained in or associated with the protein. In any case the maximum growth rate may not be the best in a species not raised for meat.

The key position sometimes assigned to protein in long-range discussions on human food supplies is thus transferred to amino-acids. Protein as such loses much of its significance, and differences in nutritive value between proteins become largely explicable in terms of their content of essential amino-acids; "essential", in this connexion, means amino-acids that the human body cannot synthesize, or fails to produce in adequate amounts. This change of view-point opens up new possibilities. Industrial synthesis of proteins from inorganic raw materials seems at most a remote dream; that of amino-acids from such materials as limestone, atmospheric nitrogen, and water is now possible in principle and could probably be achieved in fact using knowledge now available or obtainable by existing methods.

A large body of data already exists on the amino-acids present in proteins used for human food; it has been applied with success in blending foodstuffs of vegetable origin to give a better balance of amino-acids than any one of them could supply alone. This is possible because the limiting deficiency in different plant proteins is not always the same amino-acid (Chick, 1951, 1954; Scrimshaw, Squibb, Bressani, Béhar, Viteri, & Arroyave, 1957; de Maeyer & Vanderhorgh, 1958; Krishnamurthy, Ramakrishnan, Ganapathy, Rajagopalan, Swaminathan, Sankaran, & Subramanyan, 1959; Subramanyan, Doraiswamy, Bhagavan, Tasker, Sankaran, Rajagopalan, & Swaminathan, 1959; Tasker, Rao, Swaminathan, & Subramanyan, 1959). Schuphan (1959, 1960) showed by extensive analyses that in food plants the highest concentrations of essential amino-acids occur in the metabolically more active tissues. Protein from the banana fruit has an unusually high histidine content, an interesting example of a vegetable protein with a high proportion of an essential amino-acid (Bhagavan & Rajagopalan, 1956; Ramachandran & Phansalar, 1956). Some degree of beneficial blending occurs in any mixed diet, but its effectiveness can be increased

by intelligent use of amino acid analyses for different foodstuffs. Suitable mixtures of vegetable proteins may nutritionally replace animal protein in the human diet or at least greatly reduce the amount of animal protein needed. Vegetable proteins can also be supplemented with synthetic amino acids, the amounts correcting partial deficiencies would be small compared with those needed to replace the entire protein content of the diet. Amino acids could also be obtained by hydrolysis of plant products unsuitable for food, or difficult to convert to an edible form. Difficulties in efficient hydrolysis of protein mixed with other material, and in large scale separation of the amino acids produced, might, however, make this method less effective than direct synthesis. The latter can concentrate on the nutritionally critical amino acids, which in general form a rather small proportion of protein hydrolysates. None of the essential amino acids is as complex chemically as some vitamins now industrially synthesized, to play a significant part in world nutrition they would be needed in larger amounts than the vitamins, but their production on this scale seems practicable. The metabolic flexibility of *Chlorella* may perhaps be utilized to produce proteins containing unusually large amounts of essential amino acids. Champigny (1958b) showed that on replacement of nitrate by urea in the culture medium of *Chlorella pyrenoidosa* the amounts of soluble and protein nitrogen both increased, and the protein was richer in arginine, lysine, and leucine. Unicellular algae have interesting possibilities as economical producers of protein for direct human consumption or use as stock food if difficulties in their large scale cultivation can be overcome.

Leaves provide another potential source of protein now little used. Their protein is of high quality in terms of essential amino acids but being enclosed in cellulose cell walls is not readily accessible to animals unless their digestive equipment includes, as in ruminants, cellulose digesting bacteria. Methods have been developed for extraction of protein from herbage in a form suitable for consumption by non ruminant animals, the product could be used directly as human food, but is perhaps more likely to be used in feeding poultry or domestic animals.

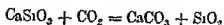
No likely assistance from synthetic products will remove the need for improvements in agricultural efficiency, in view of the increasing world population and the inadequate diets now available in many parts of the world. Output can be increased by using land not now devoted to agriculture. A reserve of unexploited land exists in some countries, but most of it offers difficulties for one reason or another.

Irrigation and correction of deficiencies in minor elements can help here, but increased yields from existing farmlands are still more desirable. Better nitrogen supplies for crops and pastures could considerably improve production. They could be obtained from synthetic nitrogen compounds, or indirectly through better growth of nodulated legumes. Much has already been done in selecting desirable host-rhizobium combinations in cultivated legumes, but great advances are still possible in this field, particularly among the tropical species, many of which have hardly been studied at all. Prospects for markedly improving the present performance of non-symbiotic nitrogen-fixing soil bacteria seem rather dim; blue-green algae, as yet little studied, probably have greater potentialities, being photosynthetic and adapted to a wide range of habitats.

It is unrealistic to consider one element alone in discussing agricultural issues. The importance of phosphorus has already been mentioned incidentally. Gnamo, and phosphate rocks derived from it, have made great contributions to agriculture over the last hundred years; many of the deposits are exhausted and the remainder will be within a period probably measured in tens rather than hundreds of years. Phosphorite deposits are more extensive, but presumably also exhaustible; they are replaced only when geological changes raise the floors of shallow seas with phosphate-rich sediments. Present techniques of agriculture disperse over wide areas of agricultural and grazing land phosphates obtained from concentrated deposits of biological origin; techniques of sanitation ensure that a large part of the phosphorus so used finally reaches the sea, which also receives phosphorus leached from the land. As the solubility of phosphates in sea water is very low, there is a steady loss of the element from the biological cycle by its deposition on the floor of the deep ocean. Phosphorus rather than nitrogen is the most likely limiting factor for biological activity in the sea.

These considerations suggest that, among the major elements needed by plants, phosphorus is the one most likely to be a limiting factor in world agriculture. Potash, deficient in many soils, could if necessary be extracted from sea water, in which its concentration is comparatively high. The low content of carbon dioxide in the atmosphere, and the vast amounts of carbon locked up during geological history in fossil fuels and carbonate rocks, might suggest carbon as a vulnerable element. On the contrary, atmospheric carbon dioxide appears to be increasing. This has been attributed to the combustion of industrial fuels, but the amounts so produced are small compared

with those used in photosynthesis and other factors may well be involved. Clearing of forests and their replacement by crops or in some cases by eroded hill sides may reduce the total photosynthesis of the earth. It may also cause a sudden release of carbon dioxide through oxidation of humus in the soils previously protected by forest. Formation of coal, lignite and petroleum particularly during the Carboniferous period may have markedly decreased carbon dioxide in the atmosphere as suggested by Brongniart (1828). A rather low upper limit to the carbon dioxide content of the atmosphere is set (Urey 1952) by reactions of the type



The use of fertilizers transported from distant sources of concentrated supplies is characteristic of modern agriculture. Another new feature is increased dependence of agriculture on power and so to a large extent on fossil fuels. This dependence existed earlier in a much smaller degree through the use of tools made from metal whose melting and fabrication needed fuel. Until comparatively recent times the fuel used was charcoal derived from timber and so readily replaceable. Today agriculture uses a wider range of tools and they require fossil fuel. Fuel is also used in considerable quantity to transport and process agricultural products. As recently as fifty years ago farming operations were powered largely by the muscles of man and his domestic animals though steam power was used on a large scale in transport and to a small extent in threshing and deep ploughing. Fishing too is now largely dependent on fuel powered vessels. This industrialization of agriculture has in a short period affected much of the world and is still spreading rapidly. It has greatly increased production per man year even allowing for employment in industries supplying equipment and fuel for agriculture. A tendency towards increased production per unit area over this period is probably due more to improved varieties and better use of fertilizers than to mechanization. The impact of new methods on the biological cycles of nitrogen and other elements is not yet clear. The disappearance of draught animals from the agricultural scene removes a source of organic manure but the effects of new methods of working the land on erosion and leaching may be more important.

## H Non-biological processes and the nitrogen cycle

The main features of the nitrogen cycle as it operates today are determined by the activities of organisms. Combined nitrogen enters



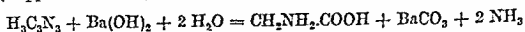
the cycle through electrical or photochemical fixation in the atmosphere; volcanic activity supplies ammonia of possibly non-biological origin. Photochemical nitrification may occur in the soil (Dhar, Bhattacharya, & Biswas, 1933; Corbet, 1934) though it is unlikely to be as important as bacterial nitrification. Ultra-violet light induces several changes in dissolved nitrogenous compounds, converting both ammonia and nitrate to nitrite, and liberating molecular nitrogen from ammonium nitrite (Bertelot & Gaudichon, 1911). A rapid mineralization of organic nitrogen to ammonia and to a lesser extent to nitrate has been observed in the upper layers of very dry soil in hot weather (Lebedyantsev, 1924; Drouineau, Lefèvre, & Blane-Aicard, 1953). The French workers found up to 100 kg N/ha/month to be mineralized in this way in localities near the Mediterranean. Soil temperatures were so high and the moisture content (6 per cent) so low that microbiological activity seemed unlikely. Wetselaar (1960) attributed accumulation of nitrate in surface soils during the dry season in tropical Australia mainly to capillary movement from lower levels; chloride increased at the same time.

A non-biological fixation of nitrogen in the soil cannot be excluded but has never been satisfactorily demonstrated. Loew (1890b) found that in alkaline conditions nitrogen and water combined in the presence of platinum to form ammonium nitrite. Platinum is not a frequent constituent of soils; iron is, and a few scattered observations suggest though they do not establish that it too may catalyse a fixation of nitrogen. Parker (1955) found an accumulation of ammonia in iron wool, in conditions suggesting fixation; further study was difficult because the phenomenon was not readily reproducible. Francis (1925) noted that rusting iron absorbs water, carbon dioxide, and ammonia and could be considered an assembling agent for the elements required in protein synthesis. An association between iron and ammonia was recorded earlier by Austin (1787) who concluded that "whenever iron rusts in contact with water in the open air, or in the earth, volatile alkali is formed." Chevallier (1828) also found ammonia in rust, and in all of thirteen samples of natural iron oxide of varied origin. Boussingault (1829) showed it to be present in iron oxide sampled *in situ* in a mine. Vauquelin (1823) was called upon by the Paris police to investigate suspected blood stains on a sword. The presence of ammonia appeared to confirm the suspicion, but Vauquelin tested rust from other iron objects and found it constantly present. He considered that rust absorbed ammonia as such from the air, a view that subsequent work has failed either to confirm or to invalidate.

Much thought and more recently experimental study have been devoted to processes capable of forming organic compounds before organisms appeared on the earth. Giglio-Tos (1910) postulated that in the primitive ocean organic compounds formed by purely chemical processes provided a substrate for the first organisms. This view was more plausible than the earlier assumption that they must have been autotrophic, with all the complexity that autotrophy implies. It was put forward independently by Oparin in 1924, his work being greatly expanded later (Oparin, 1957). Both these workers pointed out that micro-organisms would destroy any organic substances now arising spontaneously before they accumulated to any noticeable extent. C. Darwin also noted in 1871 that "a proteine compound chemically formed . . . would at the present day be instantly devoured or absorbed, which would not have been the case before living creatures were formed" (Darwin, F., 1887). Similar views were elaborated by Haldane (1929) and by Duvillier & Desguin (1942). Several workers have reported the photosynthesis of amino acids *in vitro*, Dhar & Mukerjee (1934) obtaining them from sugars and nitrate, and Eggleston (1935) from sugars and nitrite. Bahadur (1954) improved the precision of this work by isolating aspartic acid, asparagine, glycine, and serine from the reaction products of nitrate and paraformaldehyde exposed to sunlight with iron chloride as a catalyst, several other amino-acids were detected chromatographically. Formaldehyde is formed (Sahasrabudhey & Kalyanasundaram, 1948) when a silent electrical discharge passes through a mixture of carbon monoxide and hydrogen. Bahadur, Ranganayaki, & Santamaria (1958) obtained alanine, glycine, and several other amino acids photosynthetically from gaseous nitrogen and paraformaldehyde with colloidal molybdenum oxide as a catalyst.

There is good evidence, reviewed by Oparin (1957), that a wide range of hydrocarbons arises by purely inorganic processes. Hydrocarbons under the influence of electric discharges react with molecular nitrogen. Berthelot (1868, 1869) obtained hydrogen cyanide from acetylene and molecular nitrogen using both arc and spark discharges; this compound is also formed from nitrogen and methane by arc discharges (Briner & Baerfuss, 1919, Briner, Desbaillets, & Paillard, 1938). Hydrogen cyanide synthesis from nitrogen by electric discharges was reported for ethylene and acetylene by Versteeg & Winkler (1953a, b) and for polyethylene by Weninger (1960). Cyanides can also be formed without electrical energy from nitrogen, carbon, and an alkaline carbonate. This was achieved by Desfosses (1828) and Fownes (1841),

the former citing similar results by Scheele in 1783. Hydrogen cyanide in electric discharges reacts with ethylene and other hydrocarbons to form nitriles and amines (Francesconi & Ciurlo, 1923*a, b*); urea is formed in a mixture of hydrogen, nitrogen, and carbon monoxide (Crippa & Galotti, 1929). Hydrogen cyanide in contact with mild alkali forms a trimer hydrolysing in both acid and alkaline conditions to glycine (Wippermann, 1874). The latter reaction was formulated:



Miller (1955) subjected mixtures of ammonia, hydrogen, methane, and water vapour to spark or silent discharges for several days. A complex set of amino-acids was formed, the most abundant being  $\alpha$ -amino-n-butyric acid,  $\alpha$ -aminoisobutyric acid, alanine,  $\beta$ -alanine, glycine, and sarcosine. Cultrera & Ferrari (1959) obtained serine, glycine and alanine from sodium nitrite and glycerol or other simple non-nitrogenous organic compounds exposed to ultraviolet light in solution at pH 7 and 30°C. Sulphur-containing amino-acids could arise from mercaptans formed by silent discharges acting on mixtures of ethylene and hydrogen sulphide (Losanitsch & Jowitschitsch, 1897). Fox & Harada (1958) showed that a mixture of amino-acids heated to 170°C polymerized to a protein-like product of molecular weight 4,900, containing glutamic and aspartic acids and small amounts of alanine, glycine, leucine, and other amino-acids. Adenine and possibly other purines are formed (Oró, 1960) in a solution of ammonium cyanide held at 90°C for 24 hours.

These syntheses all produce optically active compounds in racemic mixtures containing equal amounts of the two possible asymmetric forms. The presence of one particular configuration is characteristic of living matter and was long supposed to be confined to it. Asymmetric syntheses have, however, been obtained in inorganic systems. Karagunis & Drikos (1934) used circularly polarized light to perform the first total asymmetric synthesis *in vitro*; similar results are recorded by later workers, e.g. Davis & Ackermann (1945). Ostromyslenski (1908) suggested the possibility of artificial asymmetric synthesis using asymmetric crystals as catalysts. Such syntheses were later realized experimentally (Terentyev, Klabunovski, & Patrikeyev, 1950; Klabunovski & Patrikeyev, 1951) with asymmetric quartz crystals carrying a thin layer of a metallic catalyst. Inorganic agencies are thus capable, given time, of producing complex compounds containing carbon, hydrogen, nitrogen, oxygen, and sulphur. The equilibrium concentrations of organic compounds in aqueous media appear (Hull, 1960) to

be very low in the presence of ultra-violet radiation. This further emphasizes (Bernal, 1960) the necessity for some assembling agent if synthesis is to continue.

Selective production of asymmetric organic molecules from inorganic materials is also feasible. The probability of its occurrence in any given case is, however, rather low, and the combined probability that all asymmetric compounds, or even the great majority, should have the same configuration is extremely small. The observed uniformity of configuration among the amino-acids and other asymmetric compounds of existing organisms remains a strong argument for their monophyletic origin. If organisms based on D-amino-acids ever appeared on our planet, they seem to have become extinct.

Some writers give the impression of assuming that once a supply of complex organic molecules was available life appeared automatically. This naïve view merely reverses the discredited opinion that only living organisms produce organic compounds. Many hypothetical accounts of the origin of life gloss over the major difficulty by a statement that self-replicating molecules of protein and nucleic acid appeared through non-living synthesis, and by an unexplained transition became the first organisms. An inorganic crystal is a self-replicating structure which selects from solution the ions necessary to its growth, and arranges them in a definite lattice to form a predetermined structure of considerable size and precision. It is not, however, an organism by any likely definition of that ambiguous term.

Bacteria are sometimes called simple organisms, a misleading phrase suggesting an easy transition from a primitive ocean of dilute soup to organisms feeding on it and resembling those familiar to us. The apparent simplicity of bacteria reflects to a considerable extent the difficulty of studying their fine structure. Metabolically they are highly complex and more versatile than larger organisms, many of whose basic biochemical mechanisms they possess. Multicellular animals and plants have obvious structural advantages compared with their unicellular counterparts, but the metabolic sophistication associated with hormones and other adjuncts of the complex body is an advance in detail rather than in principle. We can dimly visualize the interlocking complexities involved in co-ordinated synthesis of proteins and nucleic acids; it is well to remember, if one wishes to speak of simple organisms, that our present ideas on these syntheses, complex as they are, deal only with a general process modified, in each species and perhaps in each individual, by precise and delicate control mechanisms

of whose operation we can as yet form only a vague and speculative picture.

Viruses may be regarded as much simpler organisms than bacteria. They are hardly relevant in the present connexion; they have little or no independent metabolism and grow by diverting to their own use the cellular mechanisms of the host. Their existence is thus dependent on more complex organisms. A saprophytic virus using dead organic matter might represent a truly simple stage in the evolution of organisms. Such objects are unknown but could easily escape detection if they existed; they might be like free-living microsomes, inconspicuous in form and limited in metabolism. From such structures to the simplest cell would be a great advance, of critical importance to all further evolution. Aggregation and integration of cells to form large organisms opened the way to morphological evolution; biochemical evolution may largely have been complete at the unicellular stage.

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